MOLECULAR ANALYSIS OF AN ABSCISIC ACID DEFICIENT MUTANT, *VIVIPAROUS14* OF MAIZE

BY

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MOLECULAR ANALYSIS OF AN ABSCISIC ACID DEFICIENT MUTANT *VIVIPAROUS14* IN MAIZE

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Abscisic acid (ABA), a key regulator of seed maturation, dormancy and stress responses throughout plant development, is proposed to be synthesized by oxidative cleavage of 9-*cis* epoxy-carotenoids. Carotenoid cleavage is the first committed and presumed regulatory step in ABA biosynthesis. In this study, a new ABA deficient mutant *viviparous14* (*vp14*) was identified in maize. Although *vp14* plants were fully viable and non-wilting in field, detached leaves of non-stressed mutant seedlings grown in the greenhouse showed markedly higher rates of water loss than the wild type. Mutant embryos exhibited normal sensitivity to exogenous ABA, and the ABA content of developing mutant embryos was 70% lower than the wild type, indicating a defect in ABA biosynthesis. The HPLC analysis of the *vp14* embryos detected no significant changes in the epoxy-carotenoid precursors of ABA, and the cell extracts of *vp14* embryos efficiently

converted xanthoxin to ABA, suggesting that *vp14* is blocked in the cleavage of *9-cis* epoxy carotenoids.

The *Vp14* gene was molecularly cloned by transposon tagging. The *Vp14* mRNA was detected in embryos and seedling roots, but not in nonstressed leaves. Southern blot analysis and cloning of related sequences indicated that *Vp14* belongs to a gene family. Several related gene sequences were cloned and the DNA sequenced. A *Vp14*-like duplicate was mapped to chromosome 5S and found to potentially encode a protein with 93% amino acid identity to VP14, suggesting that it may have a function equivalent to VP14. The *Vp14* mRNA and related transcripts were induced by water stress in leaves. The VP14 amino acid sequence showed significant similarity to lignostilbene dioxygenase of bacteria and RPE65, a protein found in the mammalian retinal pigment epithelium. Purified recombinant VP14 as expressed in *E. coli* specifically cleaves 9'-*cis*-neoxanthin/9-*cis*-violaxanthin to produce xanthoxin and a C_{25} allenic/epoxy apo-aldehyde. The implications of this demonstration of specific enzymatic cleavage of carotenoids for understanding the mechanism of vitamin A biosynthesis in mammalians are discussed. Evidence that the VP14 protein is localized in the chloroplast of plant cells was obtained by an *in vitro* chloroplast protein import assay. Precursor VP14 was imported into a soluble fraction of chloroplast lysate and processed. A significant amount of imported VP14 was also found associated with thylakoid membrane with a topology of facing stroma. Furthermore, VP14 was subject to a fast turnover with a half life of about 30 minutes, suggesting that degradation may play a role in controlling the enzyme abundance.

CHAPTER 1
REVIEW OF LITERATURE

The discovery of the plant hormone, abscisic acid (ABA), was associated with two independent lines of research, one involved isolation of substances which accelerate leaf abscission in cotton by Addicott's group, the other included a search for substances which cause bud dormancy in woody plants by Wareing's group. The search for an abscission accelerator from young cotton fruits identified a compound "abscisin II" (Ohkuma et al. 1963). The search for a dormancy inducer from *Betula pubescens* led to the discovery of a substance, named "dormin" (Eagles and Wareing, 1964). Soon thereafter, the two substances were found to be chemically identical in structure (Cornforth et al. 1965). Since then, a uniform name was given as abscisic acid. The naturally occurring ABA is the S-enantiomer. Whereas the R-enantiomer has been reported to be biologically active in most cases, it is inactive in regulating stomata aperture (Addicott 1983). The biologically active configuration of ABA is 9-*cis* ABA, and *all trans* ABA is biologically inactive.

ABA Synthetic Pathways in Fungi and Plants

Although its structure was elucidated more than 30 years ago (Cornforth et al. 1965), the ABA biosynthetic pathway had remained elusive until the late 1980's. Studies of the ABA biosynthetic pathway in higher plants have encountered great difficulties compared with the similar studies on other plant hormones such as ethylene and

cytokinins. ABA exists at very low concentrations (10^{-7} - 10^{-8} M), even in water stressed tissues and maturing embryos where it is around 10^{-6} M. Thus, analysis of ABA was technically challenging. Furthermore, the major obstacle is the poor incorporation of isotope labeled precursors such as ^{14}C-MVA (mevalonic acid) and $^{14}CO_2$ into the intermediates and ABA such that one of the most important approaches to studying metabolic pathways has been of little use in studying ABA biosynthesis (Zeevaart and Creelman 1988, Walton and Li 1995). The poor incorporation of ^{14}C-MVA into ABA led to a number of assumptions about precursor pools and localization of ABA synthesis. Milborrow (1983) suggested that ABA may be synthesized in the chloroplast; thus, the fed precursors have to cross two layers of chloroplast membrane to reach ABA synthetic enzymes. In fact, currently it is widely believed that ABA is synthesized in plastids. Another explanation is the existence of a relatively large pool of precursors such that the incorporated labeled precursor was largely diluted. Alternatively, the pool used for ABA biosynthesis may be separated from the one reached by conventional feeding experiments. Some of those assumptions turned out to be correct as now we know that ABA is derived from an oxidative cleavage of carotenoids which exists abundantly and exclusively in plastids of higher plants.

The Direct C_{15} Pathway of ABA Synthesis in Fungi

The discovery in 1977 that a rose pathogen, the fungus *Cercospora rosicola*, produces and excretes relatively large quantities of the naturally occurring enantiomer of ABA into its growth medium initiated work on the biosynthetic pathway in that organism (Asante et al. 1977). The hypothesis was then very simple that higher plants might use the same pathway as in fungi. Radioactive isotope labeled MVA was fed to *C. rosicola*

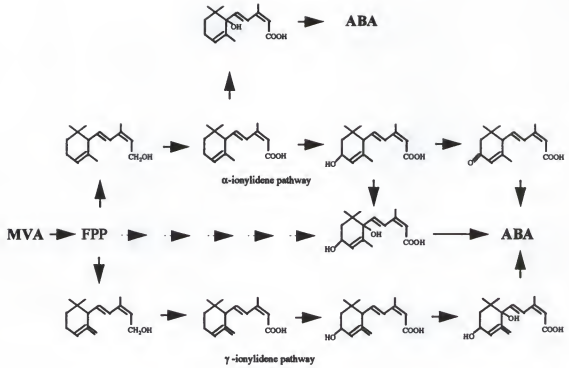


Fig. 1-1. The direct ABA biosynthetic pathway found in the fungi *Cercospora rosicola* (α-ionylidene pathway), *C. cruenta* (γ-ionylidene pathway), *C. pini-densiflorae*, and *Botrytis cinerea*. A single arrow between two compounds does not necessarily indicate that only one enzyme step is involved. (Adapted from Zeevaart and Creelman 1988)

and shown to be converted to 1'-deoxy-ABA, a major accumulated intermediate. Purified 1'-deoxy-ABA fed to the fungus was converted to ABA with a good yield. Analysis of the labeled-intermediates in *C. rosicola* fed [1,2-^{13}C]-acetate revealed that ABA is synthesized *via* an isoprenoid pathway as indicated in Fig. 1-1 (Bennett et al. 1981, 1984). The radioisotope labeled intermediates were followed a chasing pattern, MVA, farnesyl pyrophosphate (FPP), α-ionylidene, α -ionylidene ethanol, α-ionylidene acetic acid, 4'hydroxy-ionylidene acetic acid, 1'-deoxy-ABA in *C. rosicola*.

There seemed to be variations in other fungal species regarding ABA synthesis, because similar assays revealed different radio-labeled intermediates. The major difference may be the order of the hydroxylation and oxidation of ionylidene. In *B. cinerea*, 1', 4'-t-diol of ABA was shown to be the immediate precursor of ABA (Hirai 1986). In C. *pini-densiflоorae*, ABA seems to be derived from 4'-hydroxy-ionylidene acetic acid *via* 1'4'-t-diol of ABA because the latter can be converted at a higher rate (Okamoto et al. 1987). In *C. cruenta*, the radio-labeled MVA was converted to ABA in the following order, γ-ionylidene ethanol, 4'-hydroxy-r-ionylidene acetic acid, 1',4'-dihydroxy-γ-ionylidene acetic acid and ABA (Oritani et al. 1985, Oritani and Yamamoto 1985). Although different fungal species may use one or more of the suggested pathways, all the intermediates have 15 carbons and are structurally analogous to ABA. Fluridone, a carotenoid synthesis inhibitor, did not inhibit ABA synthesis in *C. rosicola* indicating carotenoids are not involved in the ABA biosynthesis (Oritani and Yamamoto 1985).

In a search for a similar pathway in higher plants, tissues from a variety of plant species were fed with ^{14}C-MVA (Zeevaart and Creelman 1988). In contrast to fungi, only a very low incorporation into ABA was detected. Feeding of bean and avocado fruits

with ionylidene or 1'-deoxy- ionylidene did not produce ABA as was observed in fungi (Zeevaart and Creelman 1988). The only report that showed ABA could be directly converted from C_{15} substrates was provided by Robertson (Milborrow 1983), in which ^{14}C-phytoene and ^{3}H-MVA were fed to avocado fruits. Both ^{14}C and ^{3}H were found in β-carotene, but only the ^{3}H label was found in ABA. This experiment was later regarded as inconclusive because the possible existence of a separate β-carotene pool that is inaccessible to ABA synthetic enzymes was not ruled out and the C^{14} incorporation of into xanthophylls was not examined (Zeevaart et al. 1989).

The Indirect C_{40} Pathway of ABA Synthesis in Higher Plants

For about thirty years since the elucidation of ABA structure, there has been a debate as to whether ABA is synthesized in higher plants *via* a direct C_{15} pathway (sesquiterpenoid) or *via* an indirect C_{40} (apo-carotenoid pathway). Because little evidence supported the direct pathway in higher plants and due to the emerging evidence in support of an indirect pathway, a universal pathway of ABA biosynthesis was proposed for higher plants (Zeevaart et al. 1989, Parry et al. 1992, Walton and Li 1992 1995). Three lines of evidence confirmed that ABA is synthesized predominantly from an oxidative cleavage of 9-*cis* xanthophylls (oxygenated carotenoids). 1). Carotenoid mutants in maize which block the synthesis of certain carotenoids also blocked the synthesis of ABA strongly indicating that ABA is derived the from carotenoid precursors. The albino, viviparous mutants of maize, *vp2, vp5, vp7,vp9, y3, y9, w3* have reduced levels of ABA in embryos and cause precocious germination of developing seeds (Robertson 1955, Neill et al. 1986). The albino phenotype presumably results from

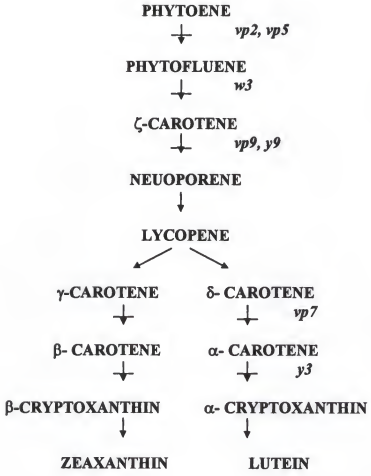


Fig. 1-2. The biosynthesis of zeaxanthin and the blocked steps of several viviparous mutants in maize. Blocked steps are marked with lines crossed the arrows for the mutants labeled on the right.

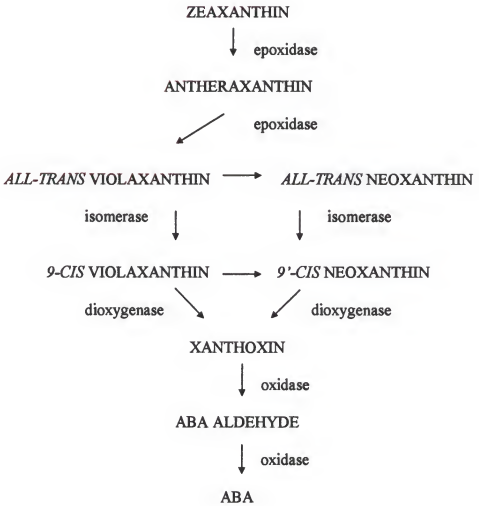


Fig. 1-3. The proposed ABA biosynthetic pathway in higher plants [Slightly modified from versions of Zeevaart and Creelman (1988) and Walton and Li (1995)].

photobleaching of chlorophylls in the absence of photoprotection provided by carotenoids. Such mutants are typically seedling lethal. Further biochemical studies have located those mutations to the carotenoid biosynthesis pathway as indicated in Fig. 1-2. 2). Incorporation of ^{18}O from O_2 into the aldehyde oxygen but not into the ring of ABA in water stressed leaves in a variety of species clearly supports an oxidative cleavage of a double bond of 9-*cis* xanthophylls (Creelman and Zeevaart 1984, Creelman et al. 1987, Li and Walton 1987, Parry et al. 1988, Gage et al. 1989; Zeevaart et al. 1989). In addition, a decrease of four xanthophylls (*all trans* violaxanthin, *all trans* neoxanthin, 9-*cis* neoxanthin and 9-*cis* violaxanthin) equals stoichiometrically to the increase of ABA and its metabolites in water stressed etiolated bean leaves (Parry et al. 1989; Li and Walton 1990a). 3). An ABA deficient mutant, *aba1* of *Arabidopsis*, was isolated by Koornneef et al. (1982) and was shown to be genetically impaired in the epoxidation of zeaxanthin to form violaxanthin (Duckham et al. 1991, Rock and Zeevaart 1991). This is an essential step for ABA biosynthesis *via* the proposed C_{40} carotenoid pathway (Zeevaart and Creelman 1988). Recently, the homologous gene of *aba1* in tobacco (*aba2*) has been cloned using heterologous transposon tagging(*Ac*) and the epoxidase activity was confirmed for the protein expressed in *E. coli* (Marin et al. 1996). In the present study, an ABA deficient mutant of maize, *viviparous14*, is shown to be blocked in the oxidative cleavage of 9-*cis* xanthophylls. The purified recombinant protein expressed as a GST-fusion in *E. coli* can cleave specifically the presumed substrates, 9-*cis* neoxanthin and 9-*cis* violaxanthin. These molecular data confirm the existence of the C_{40} pathway in higher plants. It has to be pointed out, however, that there is, so far, no conclusive evidence ruling out the existence of an alternative C_{15} pathway in higher plants.

The proposed C_{40} ABA biosynthetic pathway in higher plants is shown in Fig. 1-3. Xanthophyll cycle intermediate zeaxanthin is epoxidated to form all *trans* violaxanthin which is evidently isomerized to produce a 9-*cis* violaxanthin and 9-*cis* neoxanthin. The isomerization step is strongly supported by the fact that *all trans*-xanthoxin can not be converted to biologically active ABA by cell free extracts (Zeevaart and Creelman 1988, Walton and Li 1989). As to whether isomerization is an enzymatic or photodynamic reaction is still not clear. The existence of specifically 9-*cis* isomers, in fact, favors the enzymatic reaction view. Because 9-*cis* neoxanthin is about 10 times more abundant than 9-*cis* violaxanthin in leaves, the former is suggested to be the primary substrate for oxidative cleavage to produce xanthoxin, a compound that inhibits cell growth and has a skeleton similar to ABA. Xanthoxin is further isomerized and oxidized to produce ABA.

The oxidative cleavage reaction is the first committed step in the proposed ABA biosynthetic pathway. Substantial evidence indicates that it is also the highly regulated step of this pathway. In contrast to the endogenous level of ABA in most tissues, carotenoids and xanthophylls are far more abundant than ABA. In particular, 9-*cis* xanthophylls are found at much higher concentrations than ABA (Parry et al. 1990, Li and Walton 1990a), indicating that substrate production is not the limiting factor unless there is a separate pool for ABA biosynthesis. The endogenous level of the cleavage product, xanthoxin, is extremely low in all tissues. When xanthoxin is incubated with enzyme extracts from most tissues of bean, xanthoxin was quickly converted to ABA, indicating the enzymes downstream of xanthoxin are constitutively expressed (Sindhu and Walton 1987). Application of the protein synthesis inhibitor cycloheximide to stressed leaves reduced ABA accumulation, but did not affect the enzymatic conversion

of xanthoxin to ABA compared to the stressed leaves without cycloheximide, suggesting that those enzymes are constitutively active and not stress inducible under conditions that caused a greater than 40 fold increase in endogenous ABA level (Li and Walton 1990b, Sindhu and Walton 1990). These results indicate that the oxidative cleavage of 9-*cis* xanthophylls is likely to be the key regulated step in this pathway.

The Questions

In terms of ABA biosynthesis in higher plants, the possibility that a direct pathway may exist is not completely ruled out. The *aba2* mutant of tobacco which seems to be a null mutation due to an *Ac* element insertion into the coding region of the *Aba2* gene still retained 30% of its endogenous ABA (Marin et al. 1996). Molecular analysis indicated that *Aba2* is a single copy gene based on Southern blot analysis. Thus, the endogenous ABA in that mutant may be from one of three sources, a separate pathway like the indirect pathway, leaky mutation, or existence of redundant pathways leading to the production of violaxanthin including photodynamic conversions, *etc*. Given the important functions of ABA throughout a plant life cycle, a total knockout of ABA biosynthesis might be lethal to a plant during early embryogenesis. In that case, this class of mutants may never be isolated. The direct pathway, if it exists in higher plants, may only play a very minor role. If so, it is not surprising that mutants in such a pathway have not yet been found.

One key question with respect to the proposed indirect pathway is the demonstration of the oxidative cleavage of 9-*cis* xanthophylls to xanthoxin as it bridges the carotenoid and ABA biosynthetic pathways. Mutants impaired in other steps including zeaxanthin epoxidation and conversion of ABA aldehyde to ABA have been

found and are fairly well characterized (Taylor 1992, Rock and Zeevaart 1991, Duckham et al. 1992). Recently, a new ABA deficient mutant of *Arabidopsis*, *aba2*, was shown to be blocked in the step converting xanthoxin to ABA-aldehyde (Schwartz et al. 1997). However, no mutants that are blocked in the presumably key regulated step, the oxidative cleavage of epoxy-carotenoids, have been isolated. *Notabilis* of tomato (Parry and Horgan 1992) and *wilty* of pea (Duckham et al. 1989) which are not blocked in the conversion of xanthoxin to ABA and have normal carotenoids and xanthophylls and are thus possible candidates for mutants in the oxidative cleavage step. However, the highly leaky nature possibly due to overlapping expression of redundant genes, as discussed above, has hindered the characterization of these mutants.

Major Functions of ABA in Higher Plants

Regulation of Seed Maturation, Dormancy and Germination by ABA

ABA has been implicated in the control of many events during seed formation including maturation (Rock and Quatrano 1995), storage protein synthesis (Finkelstein et al. 1985), desiccation tolerance acquisition (Chandler and Robertson 1994, McCarty 1995), and the onset and maintenance of dormancy (Koornneef and Karssen 1994). In addition, ABA inhibits certain germination promoting processes such as expression of hydrolytic enzymes like α-amylase (Jacobsen et al. 1995), which re-mobilize the storage reserves in endosperm during germination.

Physiological studies indicated that ABA concentration peaks in developing seeds around the time of maximum fresh weight in many species just prior to the acquisition of desiccation tolerance and to onset of dormancy (Koornneef and Karssen 1994, Chandler

and Robertson 1994). Many proteins have been identified that are positively regulated by ABA in seeds, and these may play important physiological roles in developmental arrest or seed maturation. A group of seed storage proteins, the late embryogenesis abundant proteins (LEA), were found to be activated by ABA (reviewed by Rock and Quatrano 1995). Application of ABA to germinating seeds not only arrests germination, but also initiates synthesis of LEA proteins, indicating that ABA is a key signal for expression of *Lea* genes. Several *Lea* genes have been cloned and through studies of promoter structure, a conserved ABA responsive *cis*-element (ACTG core element) has been identified (Marcotte et al. 1992, Rock and Quatrano 1995). Rich in hydroxylated and hydrophilic amino acids, LEAs are believed to be osmoprotectants which help protect cells of seed tissues from desiccation (Rock and Quatrano 1995).

Genetic evidence for ABA function in seed development and dormancy was revealed by the studies of ABA deficient and ABA insensitive seed mutants. Deficiency of ABA synthesis in developing maize kernels, occurs in the *vp2*, *vp5*, *vp7* and *vp9* mutants. Such mutants cause the embryo to bypass dormancy and germinate precociously before completion of seed maturation, *i.e.* vivipary (reviewed by McCarty 1995). These mutant kernels neither develop desiccation tolerance nor accumulate certain LEAs, suggesting that ABA is absolutely required for the acquisition of desiccation tolerance, and the onset and maintenance of seed dormancy. In addition, sensitivity of embryos to ABA seems also involved in the regulation of seed development and maturation. Embryos of sprouting-resistant cereal cultivars have been shown to be more sensitive to ABA than sprouting-sensitive ones (Walker-Simmons 1987). The ABA insensitive (*abi*) mutants of *Arabidopsis abi3* (Koornneef et al. 1984), *abi4* and *abi5* (Finkelstein 1994),

and the mutant of maize *vp1* (Robertson 1965, McCarty et al. 1991) exhibit non-dormant and desiccation intolerant phenotypes, clearly indicating the function of ABA in controlling late seed development and dormancy.

ABA is a major determinant of the onset and maintenance of seed dormancy, while the plant hormone gibberellin (GA) controls the release from dormancy and initiation of germination processes. ABA and GA play their roles sequentially but also overlap each other. Thus, transition from dormancy to germination is closely related to the environmental and developmental regulation of ABA and GA biosynthesis. As an antagonist of ABA, GA induces the expression of genes that promote utilization of stored seed reserves such as hydrolytic enzymes, whereas ABA inhibits their expression. One example is amylase expression (Jacobsen et al. 1995). The early hypothesis that a balance of GA and ABA controls dormancy and germination by a similar fashion as auxin and cytokinin controls of root and shoot differentiation, may not be entirely correct as dormancy is not affected in many GA deficient mutants (Rock and Quatrano 1994).

The interaction between sources of ABA found in an embryo may be complex. Furthermore, dependence of embryo maturation and dormancy on ABA signaling appears to vary among plant species. The fact that embryos at early stages of development can be cultured *in vitro* and develop into plants without undergoing dormancy indicates that dormancy is affected by maternal factors. However, in maize, homozygous ABA deficient, viviparous kernels of the *vp5* mutant develop in the presence of genetically heterozygous maternal tissues, indicating that ABA synthesized within the kernel itself determines the maturation processes and the onset of dormancy. Robertson (1952), using chromosome T-B translocation techniques, generated kernels that were homozygous *vp5*

in the embryo, but heterozygous in the endosperm. Such embryos still displayed a viviparous phenotype. This proved that endosperm is not an essential source of embryo ABA in maize. Thus, acquisition of desiccation tolerance and dormancy of embryo are determined by the ABA synthesized in embryo itself (McCarty 1995, Rock and Quatrano 1995). It may be possible that maternal tissues surrounding the embryos generate a signal other than ABA to activate ABA synthesis in developing embryos.

In *Arabidopsis*, ABA deficient mutants are typically not viviparous unless the activity of another key regulator of seed maturation, *Abi3*, is attenuated (Koornneef and Karssen 1994). A maternal ABA effect was also suggested by the fact that *aba1*, *abi3* double mutant F2 seeds that develop with heterozygous maternal tissues were not viviparous, whereas, double mutant seeds that develop on homozygous *aba1* plants in the next generation are *viviparous* (Koornneef and Karssen 1994). The *aba2* mutant of tobacco which is homologous to *aba1* of *Arabidopsis* is also able to complete seed maturation and to develop desiccation tolerant seeds (Marin et al. 1996). Although ABA was not directly measured in *aba2* seeds, the ABA deficiency was evident in the phenotype of reduced dormancy. *Aba2* encodes the zeaxanthin epoxidase in ABA synthesis and is a single copy gene (Marin et al. 1996). Thus, the dependence of seed maturation and dormancy on ABA signaling appears to be markedly different among *Arabidopsis*, tobacco and maize.

Regulation of Stomatal Closure

Resistance to carbon dioxide intake and transpirational water loss by leaves are controlled by stomatal pores typically located on both surfaces of plant leaves. The aperture of stomatal pores is controlled by changes in the turgor of the two surrounding

guard cells. Loss of turgor, as in case of water deficit, leads to closing of stomatal pores and hence help protect leaves from further water loss. Guard cell turgor is directly related to K^+ influx and efflux which is controlled at least in part by ABA.

The action of ABA on stomatal closure is manifested in a number of ABA deficient mutants. Deficiency in ABA typically causes a wilty phenotype because of a failure of mutant plants to efficiently close their stomata. These mutants include *aba1, aba2, aba3* of *Arabidopsis*; *aba2* of tobacco; *sitiens* and *flacca* of tomato; *wilty* of pea; and *droopy* of potato (Taylor 1991). Two ABA insensitive mutants (*abi1* and *abi2*) of *Arabidopsis* also exhibit a wilty phenotype suggesting that an ABA signal transduction cascade is possibly involved in the stomatal closing (Giraudat 1995). Physiological studies indicate that stomatal opening is strongly inhibited by exogenous application of ABA onto leaves or peeled epidermis of many species (Mansfield and McAinsh 1995). In many plant species, bulk ABA levels increase dramatically in leaves exposed to water stress (reviewed by Ingram and Bartels 1996). However, when the time course is taken into account, the closing of stomata generally occurs before any significant changes of ABA content can be detected in the leaves. One possible explanation for this time discrepancy may involve a fast localized accumulation of ABA around or in guard cells, through either re-localization of ABA among leaf cells or highly localized *de novo* synthesis of ABA, *e.g.* only in guard cells or the neighboring cells. In support of this suggestion, a rapid increase in the ABA concentration of a single guard cell was shown to be correlated with the closure of stomata in stressed leaves (Harris and Outlaw 1991). However, this study did not identify the source of the ABA. These results also suggest that the dramatic accumulation of ABA in stressed leaves may have functions other than

to close the stomata. It may mediate other processes involved in plant acclimation to the environment.

The sources of ABA in leaves appear to be complex. It is now well established that ABA is synthesized in roots (Cornish and Zeevaart 1988). When growing roots come in contact with drying soil, they produce ABA in increased quantities, which enters the xylem and is transported to the leaves where it may inhibit stomatal opening (Zhang and Davies 1989). This occurs before the shortage of soil moisture causes any measurable change in the water status of the leaves. Thus it is suggested that the early stages of soil drying in field grown plants lead to the production of ABA which is transported as a chemical signal to the leaves, where it causes a reduction in transpiration and prevents a decline in water potential or a loss of turgor. When leaves are detached, the increased ABA level apparently results from the *de novo* synthesis because transcription and translation inhibitors can prevent this increase (Li and Walton 1990b, Ingram and Bartels 1996). Thus, accumulated ABA in stressed leaves apparently has at least two sources, roots and local synthesis. It is possible that in field grown plants, the sources of ABA that cause stomatal closure are determined by the severity of water deficit; mild water deficit will cause increased synthesis of ABA in roots which translocates to leaf guard cells; severe and progressive water deficit will induce ABA synthesis in the leaf itself. The study of field grown maize by Tardieu and Davies (1992) has shown that there is a good correlation between stomatal conductance and ABA concentration in the xylem sap and this may be the best above-ground indicator of the water status of the root system.

ABA modulates the activities of three major classes of ion channels, inward-, outward-rectifying K+ channels and anion channels located on the guard-cell plasma

membrane (Armstrong et al. 1995). How the ABA signal cascade functions is still unknown. When guard cells are exposed to ABA, the first detected event is an influx of positive charges leading to an initial depolarization of the membrane (Thiel et al. 1992). The influxes of Ca^{2+} and H^{+} are proposed to act as secondary messengers to activate Ca2+ sensitive and voltage sensitive anion channels. This causes long term depolarization and a massive anion efflux across the guard cell membrane (Schroeder and Hagiwara 1990). It is suggested that slow anion channels could be the rate limiting step in controlling stomata closing. Using patch-clamp techniques, it was found that the slow anion channel is strongly activated by ABA and this activation can be suppressed by the protein phosphatase inhibitor, okadaic acid. this suggests a kinase/phosphatase signal cascade (Pei et al. 1997). In the ABA-insensitive (*abi*) mutants of *Arabidopsis*, *abi1* and *abi2*, failure to close stomata is associated with loss of the slow anion channel activation caused by ABA. *ABI1* encodes a protein phosphatase 2C, a component in ABA signaling pathway (Meyer et al. 1994, Leung et al. 1994). The membrane depolarization is proposed to generate the driving force for K+ efflux through the outward-rectifying K+ channels. This in turn causes the loss of turgor in guard cells, and closing of stomata (Armstrong et al. 1995, Pei et al. 1997). Conversely, ABA also inhibits the inward-rectifying K+ channel activity (Blatt 1992, Schroeder and Keller 1992).

Guard cells of tobacco transformed with the *abi1* dominant allele have reduced ABA activation of the outward-rectifying K+ channel and also show less sensitivity of the inward-rectifying K+ channels to ABA (Armstrong et al. 1995). The presence of broad-range protein kinase antagonists, H7 and staurosporine restores the sensitivity, suggesting a kinase may be a negative regulator. A similar study of the ion channels also

indicated loss of slow anion channel activation by ABA in *abi1* and *abi2* mutants (Pei et al. 1997). ABA-induced anion channel activation and stomatal closing were suppressed by protein phosphatase inhibitors in both mutants. However, kinase inhibitors did not rescue the ABA activation of slow anion channels in *abi2* mutants, indicating that *abi2* may be an upstream component in ABA signal cascade. Since most of the ABA signaling cascade as well as ABA receptors in plasma membrane of guard cells have not been identified, the positions of ABI1 and ABI2 in that cascade remain to be resolved. There are reports that Ca2+ dependent protein kinases (CDPK1 & CDPK1a) may also be involved in the ABA signal transduction pathway (Sheen et al. 1996).

Another question that remains elusive is the location of ABA receptors which hold the key to the ABA signal transduction cascade. It is believed that they are located on the outer surface of guard cell plasma membrane sensing ABA from outside the guard cells when water deficit occurs (Hartung and Davies 1991). However, micro-injection of a physiological concentration of ABA into the cytoplasm can trigger the K+ efflux and close stomata, and the extent of stomata closing is correlated with the extent of ABA uptake by the guard cells, thus suggested the existence of internal ABA receptors (Schwartz et al. 1994). An increase of ABA content inside the guard cells is correlated with the closing of stomata (Harris and Outlaw 1991), suggesting the existence of intercellular ABA receptors.

Adaptation to Stress Environments

The survival of a plant in nature frequently requires a capacity to withstand extremes of a stressful environment including drought, extreme temperature, salinity, wounding and pathogen infection. Plants have developed two overall mechanisms to

survive, stress avoidance and stress tolerance (Chandler and Robertson 1994). Avoidance is achieved by developing specialized adaptations in architecture, such as a highly developed root system to avoid drought stress. Whereas stress tolerance depends on a combination of rapid and longer-term physiological responses to stresses, such as closing stomata to prevent from further water loss and accumulation of a class of proteins to protect cells from damage (Skriver and Mundy 1990, Chandler and Robertson 1994, Ingram and Bartels 1996).

The involvement of ABA in stress acclimation is supported by several lines of evidence: (1) ABA accumulates during stress including drought, flooding, low temperature, wounding and pathogen infection (Ingram and Bartels 1996). Wright and Hiron (1969) first detected a 40 fold increase of ABA in detached, wheat leaves. Subsequent studies have reported similar phenomena in many plant species (Ingram and Bartels 1996). The mechanism of stress-dependent ABA biosynthesis is still not fully understood (Hartung and Davies 1991). (2) Plants develop freezing tolerance when treated with ABA under nonacclimating conditions (Lang et al. 1989). The ABA deficient mutant *aba1* of *Arabidopsis* does not develop cold acclimation and this can be complemented by exogenous application of ABA (Heino et al. 1990). (3) Under drought stress conditions, ABA is shown to be required for primary root elongation, and elongation of primary roots is inhibited in ABA deficient *vp5* of maize (Sharp et al. 1994). (4) Proteins that are induced by environmental stresses and associated stimuli such as RABs (responsive to ABA) can be induced by applying ABA (Skriver and Mundy 1990, Mantyla et al. 1995).

One class of ABA proteins that are strongly induced by ABA in the late embryogenesis is the LEAs. Although functions of those proteins are still unknown, synthesis of LEAs is correlated with the acquisition of desiccation tolerance in embryos. The direct relationship between LEAs and ABA was established by ABA treatment of cultured embryos, which induced LEA expression (Ingram and Bartels 1996). Recent molecular studies have also characterized the rapid induction of RAB genes in leaves or roots. These novel genes have been isolated from several species by differential screening of cDNA libraries constructed from ABA treated leaves or roots. Some of these proteins are also expressed during the maturation phase of embryo development, thus may be functionally related to LEAs (Rock and Quatrano 1995). Both LEA and RAB proteins share one predominant feature; high hydrophilicity and a high content of uncharged and hydroxylated amino acids. Conserved domains are postulated to be functionally important in desiccation protection (Dure et al. 1989), possibly through interactions with other embryo proteins and membranes. However, the RAB17 of maize was found localized in the nucleus and is suspected to play a role in the nuclear protein transport (Goday et al. 1994).

The regulation of ABA responsive gene expression is mediated by the promoters of these genes. ABA response elements (ABRE) which contain the palindromic motif CACGTG with a G-box core element (Giuliano et al. 1988) are found in RABs and LEAs, and have been extensively studied in Em gene of wheat and the rice RAB16 gene (Skriver et al. 1991, Guiltinan et al. 1990). Recently, the modular nature of the abscisic acid response complex (ABRC) was further refined in a barley EM gene, HVA1. The promoter unit necessary and sufficient for abscisic acid (ABA) induction of gene

expression consists of a G-box and one of several "coupling elements" (CEs) (Shen et al. 1995, 1996). Different combinations of the G-box with different CEs may modulate different gene responses in different tissues.

ABA is also involved in processes that lead to the establishment of freezing tolerance. However, little is known about how ABA triggers those processes. Thus far, few mutants have been isolated that have an altered freezing tolerance phenotype. A putative freezing sensitive mutant (*frs1*) of *Arabidopsis* has a reduced cold acclimation response and this response can be compensated partially by application of ABA (refer to Quatrano et al. 1997). The molecular nature underlying this mutation should be of importance to the understanding of freezing tolerance mechanisms. (Quatrano et al. 1997)

Inhibition of Growth

Physiological studies have established that exogenous application of ABA leads to inhibition of vegetative growth. At the molecular level, ABA regulates the expression of a large number of genes especially under stressful environments. Many of these ABA up-regulated genes have been cloned by differential screening; however, their functions remains unknown. The ABA down-regulated genes have drawn little attention, although, most appear to promote cell growth. An example is the inhibition of the α-amylase promoter by synergistic interaction of ABA and VP1 (Hoecker et al. 1995).

Defense Responses

The proteinase inhibitor II (*Pin2*) is a model system for wound induced gene activation (Pena-Cortes and Willmitzer 1995). The *Pin2* gene family is the best studied examples of genes which are systematically activated upon mechanical damage of plants.

The involvement of ABA in gene activation processes following mechanical damage of the plant tissues is supported by the fact that the endogenous ABA concentration rises three- to five-fold upon wounding (Pena-Cortes and Willmitzer 1995). Exogenous application of ABA on non-wounded plants can activate the expression of *Pin2* in a pattern analogous to wounding (Pena-Cortes et al. 1991). In the ABA deficient *droopy* mutant of potato (Quarrie 1982) and *sitiens* mutant of tomato (Taylor et al. 1988, Duckham et al. 1989), wounding failed to stimulate the expression of *Pin2*, and did not increase ABA in leaves (Pena-Cortes and Willmitzer 1995). Spraying ABA on wounded *droopy* and *sitiens* leaves resulted in an activation of *Pin2*, thus strongly indicating that ABA is at least one of the signals to trigger *Pin2* gene activation upon wounding. Drought stress induced ABA accumulation does not activate *Pin2*, whereas wounding of the stressed leaves of potato and tomato activates *Pin2* transcription (Pena-Cortes et al. 1989). This implys that multiple factors including ABA may account for the wounding activation of gene expression.

Several other wound induced genes have been cloned which show a pattern of wound induction similar to *Pin2* (Hildmann et al. 1992). ABA is involved in the activation of those genes. Molecular analysis of promoters from several genes and *Pin2* revealed an ABA responsive element (ABRE) that is similar to that found in ABA regulated genes including *Leas*, *Em* and *Rab16*. However, mutagenesis of this element did not affect ABA regulated expression (Lorberth et al. 1992). This suggests that different factors in addition to the presence of ABA are necessary for the induction of this set of genes in contrast to *Em*. Consistent with the multiple functions of ABA throughout plant development, the signaling pathways for ABA that regulate transcription of

different genes are expected to be complex. In addition to ABA, jasmonic acid is also involved in the activation of related genes in response to wounding (Pena-Cortes and Willmitzer 1995).

Abscission and ABA

Although abscisic acid was initially isolated as a plant hormone that enhanced organ abscission, this nomenclature is misleading. Application of ABA to plant leaves or flowers does not result in organ abscission (Addicott 1983). Ethylene is a plant hormone that more directly controls organ abscission (Abeles et al. 1992). Application of ethylene or its precursor 1-aminocylopropane-1-carboxylic acid (ACC) strongly promotes organ abscission. The link between ABA and organ abscission may be linked to an interaction of the two hormones. It is possible that ABA is involved in the senescence processes in the separation layer cells in organs undergoing abscission (Abeles et al. 1992). ABA increases ethylene production and abscission in aged citrus organs (Riov et al. 1990). Water stress may cause leaf abscission in cotton and citrus, and inhibition of ABA biosynthesis was reported to reduce leaf abscission (Gomez-Cadenas et al. 1996).

The ABA Related Mutants in Higher Plants

ABA Insensitive Mutants

The *abi1*, *abi2*, and *abi3* were isolated by Koornneef et al. (1984), and the *abi4* and *abi5* were isolated by Finkelstein (1994). These mutants were selected by germination of EMS mutagenized *Arabidopsis* seeds on media containing ABA at concentrations that completely inhibit germination of wild type seeds. The a*bi3*, *abi4* and *abi5* mutations affect seed development specifically. The severe *abi3* alleles isolated

later, *abi3*-3 (Nambara et al. 1994), *abi3*-4 *abi3*-5 (Ooms et al. 1993), exhibit a phenotype of nondormancy and desiccation intolerance. The *viviparous1* of maize exhibits a similar seed specific phenotype, and in addition controls anthocyanin synthesis in the seed (Robertson 1955, McCarty et al. 1991). Cloning of *Vp1* indicated that it encodes a novel transcription factor (McCarty et al. 1991). It can activate *C1*, and *EM* promoters in a synergistic manner in combination with ABA (Kao et al. 1996, Hoecker et al. 1995, Hottori et al. 1992, Vasil et al. 1995), but acts as a repressor of α-amylase (Hoecker et al. 1995). A B3 domain of VP1 has a cooperative of DNA binding activity to the *sph* element of C1 promoter (Suzuki et al. 1997). The sequence of ABI3 revealed strong homology to VP1 (Giraudat et al. 1992), and thus ABI3 is considered an ortholog of VP1(McCarty 1995). Unlike *abi3*, plants of *abi1* and *abi2* display a wilty leaf phenotype in normal growth conditions (Koornneef et al. 1984, Giraudat 1994). ABI1 encodes a 2C type of protein phosphatase (Meyer et al. 1994, Leung et al. 1994). ABI1 is suggested to be a signal transduction component involved in many processes including embryogenesis, ABA responses in guard cells (Amstrong et al. 1995, Pei et al. 1997) and mitotic cell division in roots (Leung et al. 1994). A puzzle remains as to how a missense mutation (Gly-180 to Asp) in the mutant *abi1* allele gives rise to a dominant phenotype. A search for *abi1* homologous sequences, surprisingly, led to the cloning of *Abi2*, and the mutation in *abi2* was exactly as in *abi1*, a Gly-180 to Asp (Leung et al. 1997). Thus, ABI1 and ABI2 encode very similar protein phosphatases.

Carotenoid Biosynthetic Mutants

In maize, several viviparous mutants are associated with an albino phenotype as indicated by *vp2*, *vp5*, *vp7*, *vp9*, *w3*, *y9*, *etc*. One function of the carotenoids is to protect

the photosynthetic apparatus from photobleaching in a high light situation. Blocking the synthesis of certain carotenoids causes these mutants to be albino and generally lethal. The steps blocked in the various mutants are shown in Fig. 1-1. As ABA is synthesized from cleavage of carotenoids, certain carotenoid mutants are also ABA biosynthetic mutants. Those mutants provided key evidence that ABA is derived from carotenoids but have generated little information about the committed (specific) steps of the ABA biosynthetic pathway (Zeevaart and Creelman 1988).

ABA Biosynthetic Mutants

A number of ABA deficient mutants have been isolated from a variety of plant species as indicated in Table 1. Most of those mutants (*flc*, *sit*, *droopy*, *ckr1*, *iba1*, *etc.*) affect the last step of ABA biosynthesis, the oxidation of ABA-aldehyde to produce ABA. The tomato mutant, *Notabilis,* is less well characterized largely due to its leaky nature, but is suspected to be impaired in the oxidative cleavage of 9-*cis* neoxanthin to xanthoxin (Parry et al. 1988, Parry and Hogan 1992a, b, Taylor et al. 1988). Similarly, a pea *wilty* (*wt*) ABA deficient mutant is suspected to be blocked in the same step, since it can convert ABA aldehyde to ABA (Duckham 1989). No differences in xanthophyll and carotenoid profiles were found in *wt* mutant and wild type plants (Duckham et al. 1991). More detailed reviews including these mutants can be found elsewhere (McCarty 1995, Rock and Quatrano 1995, Taylor 1991). The following summarizes the current advances in this area.

The *Arabidopsis aba1* was isolated in a screen for revertants of the GA deficient *ga1* mutant population (Koornneef et al. 1982). *Aba1* is impaired in the conversion of zeaxanthin to violaxanthin (Duckham et al. 1991, Rock & Zeevaart 1991). The *Aba2*

mutant of tobacco was isolated by heterologous *Ac* tagging in tobacco and shown to encode zeaxanthin epoxidase by expression in *E. coli*. (Marin et al. 1996). The tobacco *aba2* gene is able to complement the *aba1* phenotype of *Arabidopsis* through T- DNA transformation, indicating that it is an otholog of *aba1* (Marin et al. 1996). Additional

Table 1-1. Abscisic acid deficient mutants

Species	Mutant	Phenotype	Blocked step	References*
Arabidopsis thaliana	*aba1*	nd, wilty, red. height	Zeaxan -> Vioxan	Koornneef 1982; Rock 1991
	aba2	red. dormancy, wilty	Xan -> ABA-ald	Leon 1996; Schwartz 1997
	aba3	red. dormancy, wilty	ABA-ald -> ABA	Leon 1996; Schwartz 1996
Nicotiana plumbaginifolia	*aba2*	nd, wilty	Zeaxan -> Vioxan	Marin 1996
(tobacco)	*clr1*	cytokinin R, wilty	ABA-ald -> ABA	Parry 1991
	iba1	auxin R, wilty	ABA-ald -> ABA	Bitoun 1990
Lycopersicon esculentum	*flacca*	wilty	ABA-ald -> ABA	Parry 1988
(tomato)	*sitten*	wilty	ABA-ald -> ABA	Parry 1988
	notabilis	slightly wilty	?	Taylor 1988; Parry 1991
Solanum phureja (Potato)	*droopy*	wilty	ABA-ald -> ABA	Duckham 1989
Pisum sativum (pea)	*wilty*	slightly wilty	?	Parry 1991
Hordeum vulgare (barley)	*nar2a*	wilty	ABA-ald -> ABA	Walker-Simmons 1989
Zea may (maize)	*vp*2274*	viviparous	this study	McCarty (1995)
	(vp14)			

Note: nd, nondormant; red., reduced; R, resistance.

* Only the first author was listed to save space.

Arabidopsis ABA deficient mutants were isolated in a screen for seed able to germinate in the presence of the gibberellin biosynthesis inhibitor paclobutrazol. Two new ABA

deficient mutants were reported that have reduced dormancy and excessive rate of leaf water loss (Leon-Kloosterziel et al. 1996). Biochemical characterization indicated that *aba2* is blocked in the conversion of xanthoxin to ABA-aldehyde and *aba3* blocked in the last step of ABA biosynthesis, *i.e.* the conversion of ABA aldehyde to ABA (Schwartz et al. 1997).

Candidates for Mutations in the Cleavage Step

Two ABA deficient mutants, *wilty* and *notabilis* of pea and tomato, are blocked at undetermined steps in ABA biosynthetic pathway (Taylor 1991). Biochemical analysis of the two mutants did not detect any defects in either the xanthophyll profiles or the conversion of xanthoxin to ABA (Parry and Horgan 1992a, b, Taylor 1991). However, the highly leaky nature of both mutations greatly compromised any conclusions based on these data. Several newly isolated viviparous mutants of maize may potentially provide new information about the pathway (McCarty 1995). Viviparous mutants of maize, characterized so far, are all associated with ABA, either through perception or synthesis. Thus, mutants with normal carotenoids but decreased ABA levels are potentially ABA biosynthetic mutants. One mutant, named *vp14-2274* (McCarty 1995), was analyzed in this study and is shown to be impaired in the oxidative cleavage step, the conversion of 9-*cis* xanthophylls to xanthoxin. Further study of these mutants is expected to further improve our understanding of ABA function and biosynthesis in plants.

Summary and Future Perspectives

ABA, a sesquiterpene plant hormone, has multiple functions throughout the plant life cycle (Addicott 1983, Zeevaart and Creelman 1988, Taylor et al. 1992, Walton and Li

1995). ABA plays important roles in the inhibition of growth, control of stomatal aperture, organ abscission, embryo development, dormancy, tolerance of stresses and plant defense responses (Zeevaart and Creelman 1988, Hartung and Davies 1991, Black 1991, Prescott and John 1996). In addition, ABA is particularly interesting with regard to its molecular regulation because hormone levels increase 10- to 50-fold within hours of environmental perturbations (Walton and Li 1995). Considerable effort has been directed toward dissection of the ABA biosynthetic pathway since elucidation of the chemical structure of ABA in 1965 (Addicott). We now know that in certain fungi (*Cercospora rosicola, etc*) ABA is synthesized from a C_{15} isopropenoid *via* a so called direct pathway (Bennett et al. 1984, Zeevaart and Creelman 1988). In contrast, higher plants synthesize ABA largely from a C_{40} carotenoid (zeaxanthin) *via* a so called indirect pathway using an oxidative cleavage of *9'-cis* xanthophylls to produce xanthoxin. Xanthoxin is converted through two additional steps to ABA (Zeevaart and Creelman 1988, Walton and Li 1995). The cleavage step is the first committed step leading to ABA biosynthesis and substantial evidence has indicated that it is likely to be the key regulated step in ABA biosynthesis (Zeevaart and Creelman 1988, Walton and Li 1995). The epoxidase which converts zeaxanthin to *all-trans* violaxanthin has recently been cloned in tobacco and the enzyme activity was confirmed (Marin et al. 1996). However, none of the enzymes that are specific to ABA synthesis have been isolated, particularly the hypothesized dioxygenase that cleaves carotenoids.

Mutants have proven to be very powerful in the elucidation of biosynthetic pathways, particularly in the case of ABA biosynthesis, where conventional isotope labeling has not been feasible. A single recessive mutation such as *vp5* has provided

important evidence that ABA is synthesized from carotenoids. The *aba1* mutant of *Arabidopsis* and *aba2* of tobacco have led to the conclusion that zeaxanthin is a precursor of ABA synthesis and that an epoxidation of zeaxanthin to form violaxanthin is required for ABA synthesis (Rock and Zeevaart 1991, Buckham et al. 1992, Marin et al. 1996). Other mutants like *sittens*, *flacca*, *etc.* have confirmed the last step of ABA synthesis (Taylor 1992). Recently, an ABA deficient mutant of *Arabidopsis*, *aba2*, was shown to be blocked in the step of converting xanthoxin to ABA-aldehyde (Schwarts et al. 1997). However, mutants that block other intervening steps in the ABA synthetic pathway are still lacking, particularly mutants blocking the proposed oxidative cleavage of epoxy-carotenoids. Mutants that potentially regulate ABA biosynthesis are also required. Thus, successful isolation of new, developmentally specific ABA deficient mutants provide an important way to the study of ABA biosynthesis and regulation (McCarty 1995). Eventually, the combination of molecular cloning, genetics, biochemistry and physiological studies will lead to a better understanding mechanisms for ABA action in plants. This will include developmental and environmental regulation of ABA synthesis, as well as how ABA regulates the expression of other genes to exert its function as well. Agricultural applications resulting from manipulation of ABA synthesis in crops will also be possible.

Mutants are also required for explanation of the ABA signal transduction cascade since it is the key to understanding how ABA functions in plants. ABA signal transduction pathways are likely complex, as indicated by the functions of ABA throughout plant life cycle. Potential components of the ABA transduction pathway such as *Abi1* and *Abi2* have begun to emerge (Meyer et al. 1994, Leung et al. 1994 1997). A

recent mutant in *Arabidospsis* which confers an enhanced response to ABA (*era*) encodes the beta subunit of farnesyl transferase, a putatively negative regulator of ABA signal transduction pathway (Cutlers et al. 1996). However, the key details of how the ABA signal is perceived and transduced in plant cells are far from clear.

CHAPTER 2
MOLECULAR ANALYSIS OF *VIVIPAROUS14*, A DEVELOPMENTALLY SPECIFIC ABSCISIC ACID BIOSYNTHETIC MUTANT OF MAIZE

Introduction

The plant hormone, abscisic acid (ABA) plays a key role in the regulation of seed maturation and dormancy (McCarty 1995, Rock and Quatrano 1995) and mediates plant responses to a variety of stress conditions including wounding (Pena-Cortes and Willmitzer 1995), drought (Vartanian et al. 1994, Giraudat 1995) and cold acclimation (Gilmour et al. 1991). ABA is synthesized from carotenoids as manifested in the ABA deficient mutants of maize (e.g. *vp2*, *vp5*, *vp7*, and *vp9*) that are in fact blocked in the early steps of carotenoid synthesis (Neill et al. 1987). Biochemical studies (reviewed by Zeevaart and Creelman 1988) and analyses of mutants that block ABA synthesis (reviewed by Taylor 1991) have led to a proposed indirect ABA synthetic pathway in higher plants (refer to Chapter 1, Zeevaart and Creelman 1988, Walton and Li 1995). The first committed step in the pathway is the cleavage of two potential substrates, 9-*cis* violaxanthin and 9-*cis* neoxanthin to form xanthoxin which is subsequently converted to ABA *via* an ABA-aldehyde intermediate.

Mutants blocked in ABA synthesis have been isolated in a variety of plant species including maize, *Arabidopsis*, tomato, tobacco, potato, etc. (Robertson 1955, Koornneef at al. 1984, Leon-kloosterziel et al. 1996, Marin et al. 1996, Neill et al. 1987). ABA deficient mutants of maize are viviparous, *i.e.* developing kernels fail to enter

developmental arrest in the mid to late stage of seed maturation and germinate while still attached to the plant (Neill et al. 1986 1987, Robichaud et al. 1987). The viviparous mutants of maize, *w3*, *y3*, *vp2*, *vp5*, *vp7* and *vp9* are blocked in early steps of carotenoid biosynthesis, and thus also display albino (carotenoid deficient) and seedling lethal phenotypes due to photobleaching of chlorophyll (Neill et al. 1986, refer to Fig.1-1 for blocked steps). These mutants are not considered specific to ABA synthesis and have contributed little to the studies of the ABA specific portion of the biosynthetic pathway (Zeevaart and Creelman 1988). ABA deficient mutants in other species (tomato, *Arabidopsis*, tobacco, etc) are generally not viviparous indicating that dependence of seed maturation on ABA signaling varies markedly among these species (McCarty 1995). The *aba1* mutant of *Arabidopsis* exhibits enhanced sensitivity to water stress and a reduced requirement for gibberellin hormone synthesis during seed germination (Koornneef et al. 1986, 1989). *aba1* is deficient in the epoxy carotenoids, such as neoxanthin and violaxanthin, due to a genetic lesion in the epoxidation of zeaxanthin (Rock and Zeevaart 1991, Duckham et al. 1991). The *aba2* mutant of tobacco, recently isolated and cloned by transposon (T-DNA/Ac) tagging, is homologous to *aba1* of *Arabidopsis* and encodes a zeaxanthin epoxidase (Marin et al. 1996). The *flacca* and *sitiens* mutants of tomato (Sindhu and Walton 1988), and the *aba2* and *aba3* mutants of *Arabidopsis* (Schwartz et al. 1997) genetically define two steps downstream of the cleavage reaction that allow conversion of xanthoxin to ABA. Thus far, mutants blocked in several steps of the pathway have been isolated. None of these mutants are known to be blocked in a key step that connects C_{40} epoxy-carotenoids and C15 xanthoxin, *i.e.* the cleavage of epoxy-carotenoids. The *notabilis* mutant of tomato is the only reported candidate for the

carotenoid oxidative cleavage (Parry et al. 1992 a, b). However, the blocked step has been biochemically difficult to locate in *notabilis* largely as a result of its highly leaky nature.

Circumstantial evidence indicates that the oxidative cleavage of epoxy-carotenoids is the key regulated step in the ABA biosynthetic pathway. The carotenoid precursors of ABA are widely distributed in plant tissues. Epoxidation of zeaxanthin was ruled out to play a regulating role in ABA synthesis (Marin et al. 1996). The potential immediate precursors in the proposed reaction (Creelman et al. 1992, Walton and Li 1995), 9-*cis* violaxanthin and 9-*cis* neoxanthin, are far more abundant than the endogenous ABA levels in leaves of maize, pea, tomato and other species (Parry et al. 1990, Li and Walton 1987). This indicated that steps prior to formation of the 9-*cis* xanthophylls are unlikely to limit ABA synthesis. The enzyme activities downstream of the oxidative cleavage step required for conversion of xanthoxin to ABA also appear to be constitutively active in most plant tissues (Sindhu and Walton 1987). The enzymatic activities required for conversion of xanthoxin to ABA, as assayed *in vitro*, are not regulated by drought stress, and are also not inhibited by protein synthesis and transcription inhibitors in stressed leaves (Li and Walton 1990). Accumulation of ABA by stresses can be inhibited by inhibitors of both transcription and translation in a variety of species (Ingram and Bartels 1996). These studies have led to the hypothesis that the oxidative cleavage of 9-*cis* xanthophylls to form xanthoxin is the key regulated step in the ABA synthetic pathway (Sindhu and Walton 1987, Creelman et al. 1992).

As suggested by McCarty (1995), a potentially important approach for identifying mutants in regulated steps of ABA synthesis is to look for mutants that affect ABA synthesis in specific organs or phases of development. In maize, evidence that all the

known ABA deficient mutants are viviparous indicated that vivipary is possibly a sensitive seed specific phenotype for detection of ABA deficient mutants. To generate clonable ABA deficient mutants in maize, we screened active *Robertson's Mutator* transposon populations for viviparous seed mutants. To avoid mutants that were blocked in carotenoid biosynthesis, we specifically looked for mutants that were viviparous but fully viable as homozygotes and had normal carotenoid pigmentation (McCarty 1995). Here we report evidence that one of the ABA deficient mutants, *viviparous14* (*vp14*), is blocked in the cleavage step of epoxy-carotenoids. Molecular analysis of the *Vp14* gene indicates that it encodes a protein related to lignostilbene dioxygenase, a bacterial enzyme which catalyzes a double bond cleavage reaction with striking similarity to the carotenoid cleavage step of ABA biosynthesis. We show that *Vp14* and related genes are developmentally regulated and induced in leaves by drought stress.

Materials and Methods

The *vp14*-2274 and *vp14*-3250 mutants were identified in active Robertson's Mutator lines. Wild type W22, M14, Q66, Q67, Q77, Q79 strains that founded the Mutator population were a gift of Donald S. Robertson, Iowa State University. The nonsegregating wild type NS-2274 and homozygous *vp14-2274* mutant strains used for biochemical and molecular analysis were extracted from a segregating *vp14*-2274 stock that had been maintained by self pollination and selection of heterozygous plants.

ABA Determination and HPLC Analysis of Carotenoids and Xanthophylls

Embryos were harvested in Florida at 16, 18 and 20 days post-pollination and frozen in liquid nitrogen. Extraction, purification and analysis of carotenoids and xanthophylls by

HPLC was carried out in collaboration with Dr. Jan Zeevaart's group at Michigan State University using methods described earlier (Rock and Zeevaart 1991). ABA extractions, purification, and quantification by GC-MS with electron capture detector (ECD) were performed as described (Leon-Kloosterziel et al. 1996b).

Determination of ABA Sensitivity in Culture Ears were harvested from green house grown plants at 16 and 18 days after pollination (DAP) and surface sterilized by submersion in a 70% ethanol solution containing 1% dish detergent for 5 minutes. Embryos were harvested aseptically and placed in magenta boxes containing MS medium (pH 5.7), 0.2% phytagel and the indicated concentration of abscisic acid (Robichaud and Sussex 1986). Embryos were cultured at 25 °C in a growth chamber and shoot, root length and fresh weight were measured after four days. The data shown are the means of 15 to 20 16 DAP embryos for each treatment. 18 DAP embryos showed a similar response.

Southern and Northern Blot Analysis Genomic DNA was isolated from maize seedling leaves as described by Dellaporta (1983). Approximately 10 μg of DNA was digested with the indicated restriction enzymes and resolved by agarose gel electrophoresis. The gel was denatured then blotted onto nylon membrane (Sambrook et al 1989). The membrane was UV-linked and hybridized in a Hybaid Chamber (Bio-Rad) according to the method of Church and Gilbert (1984). The membranes were washed in a 40 mM sodium phosphate buffer (pH 7.2), 1% SDS solution at 65 °C for 2-3 hours for moderate stringency and at 70 °C for high stringency.

Total RNA was extracted from maize tissues (1-2 g) in TriZol solution according to manufacturers instructions (BRL) and purified further by precipitation with

isopropanol. Poly(A)$^+$-enriched RNA was prepared using PolyATtract according to manufacturers instructions (Promega) and quantified spectrophotometrically. 1 μg of Poly(A)$^+$-RNA was resolved in a 1.2% agarose gels containing formaldehyde (Sambrook et al. 1989). After transfer, nylon membranes were UV-linked and probed as described for southern hybridization. The probes were radiolabeled with the Random Primer DNA Labeling System (BRL) in the presence of ^{32}P-α-dCTP (3000 Ci/mmol, DuPont).

Construction and Screening of Genomic and cDNA Libraries Approximately 100 μg genomic DNA prepared from homozygous mutant or wild type plants (*vp14-2274, vp14-3250*, wild type) was digested with appropriate restriction enzymes, then size fractionated by centrifugation at 85,000 ×g at 4 °C for 24 hours through a 10-40% linear sucrose gradient prepared in sterile TE buffer (pH 8.0). Fractions containing the fragment of interest were confirmed by Southern blotting and the concentrated DNA was ligated into an appropriate lambda phage cloning vectors (λ-gt10 was used to clone the 2.5 kb xho1 fragment, λ-ZAP was used to clone the EcoRI fragments). The ligated DNA was packaged into lambda phage according to the manufacture's instructions (Stratagene).

A wild type embryo cDNA library was constructed in λ-Zap from 5 μg of poly(A)$^+$-mRNA prepared from 18 day post pollination inbred W22 embryos. cDNA was prepared and cloned using the λ-ZAP Express cDNA Synthesis Kit according to the manufacture's instruction (Stratagene). Lambda phage libraries were plated, lifted on nylon membrane and probed by DNA hybridization as described above for Southern and northern blots.

A 2.5 kb XhoI *Mu1* containing genomic fragment isolated from *vp14-2274* was subcloned in pBluescript (Promega) and PCR was used to amplify and clone a 1 kb

sequence that flanked the *Mu1* insertion element. A oligonucleotide primer specific for the inverted terminal repeat of *Mu1* was used as the 5' primer (5'-CCATAATGGCAATTAT-CTC-3') and the T7 sequencing primer for the vector was used as the 3' primer (TAATACGACTCACTATAGGG-3'). The resulting ~1 kb fragment was subcloned to pBluescript-SK.

Conversion of Xanthoxin to ABA in Cell Free Enzyme Extract This assay was performed by Dr. Zeevaart's group at Michigan State University on embryos prepared in Gainesville. Embryos were dissected from self-pollinated ears of homozygous mutant and wild type segregants of an F3 family. Embryos were homogenized in 0.2 M potassium phosphate (pH7.5) and 10 mM DTT and the extract was centrifuged to remove insoluble material. The supernatant soluble protein fraction was desalted using a G-25 Sephadex spin column. Enzyme assays contained 1 mM PMSF, 0.25 mM EDTA, 1 µg 9-*cis* xanthoxin and the crude enzyme extract. The amount of ABA produced was determined by GC-MS.

Results

Isolation of *Viviparous14* Mutant

All of the characterized viviparous mutants of maize (*vp1*, *vp2*, *vp5*, *etc.*) are blocked either in the perception or in the synthesis of ABA (McCarty et al. 1991, Neill et al. 1987). A search for viviparous mutants in maize was considered to be an efficient way to isolate ABA deficient mutants. In a screen of the maize strains containing active *Robertson's Mutators* transposons, two alleles of a new recessive mutant, *viviparous14(vp14)*, were identified and designated as *vp14-2274* and *vp14-3250*. The

vp14 mutant kernels have a weak viviparous seed phenotype. In field growth conditions, the embryo shoot axis of the mutant seeds typically elongates but frequently does not rupture the pericarp to initiate precocious germination. Thus, most mutant seeds are desiccation tolerant and are capable of germinating. The penetrance of the viviparous phenotype is variable from season to season. In the green house growth conditions where plants were well watered, the penetrance was relatively higher, as shown in Fig. 2-1, and most of the *vp14-2274* kernels that grew under these conditions also germinated fully to form shoots and roots. Some shoots were strong enough to break the pericarp while others were restrained by the pericarp. Unlike *vp1* (McCarty et al. 1989), anthocyanin pigmentation is not affected by the *vp14* mutation, and if rescued, these germinated kernels can develop into healthy, normal plants (Fig. 2-1B).

The B-A translocations in maize allow recessive genes to be located on the correct chromosome arm in the F1 population. At the second pollen division, the supernumerary B chromosome frequently nondisjoins so that one sperm cell of a pollen grain has two B chromosome and the other has none. Various maize T-B lines were created such that the B chromosome carries a specific segment translocated from an A chromosome. If a mutant is located on a translocated segment, a cross between a mutant and the specific T-B line will produce some embryos displaying the mutant phenotype in F1 because nondisjunction will produce hemizygous embryos deficient in the tested wild type gene. In crosses using homozygous *vp14-2274* and *vp14-3250* as female to a pollen parent that carried the T-B1La B-chromosome translocation (Birchler 1995), a similar

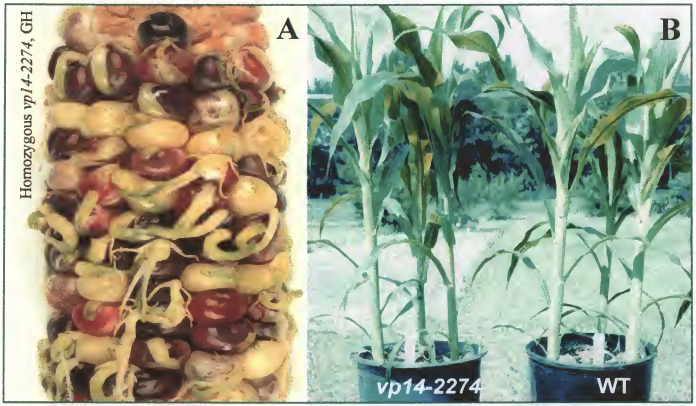


Fig. 2-1. The phenotype of *vp14*. **A.** The vivipary of a homozygous *vp14-2274* ear grown in green house. **B.** These viviparous kernels were rescued before desiccation and developed into healthy plants. The wild type plants were non-segregating *Vp14*.

viviparous phenotype was uncovered, indicating that *vp14* is located on the long arm of chromosome 1. To resolve the relationship of *vp14* relative to other viable viviparous mutants of maize, homozygous *vp14-2274* plants were crossed to *vp8* (located on 1L, Robertson 1955), *vp10* (Smith and Neuffer 1992) and other unmapped, though phenotypically similar viviparous mutants including *vp*3286* and *vp*3239* (McCarty 1995) . The *vp14-2274* mutant complemented each of these mutants, indicating that *vp14* is a new locus. It was thus named *viviparous14* (*vp14*).

vp14 Is a Developmentally Specific ABA Deficient Mutant

In the relatively harsh Florida Summer field growth conditions, the *vp14* mutant plants showed no discernible phenotype compared to wild type plants (Fig. 2-1B). In contrast to other known ABA deficient mutants such as *aba1* of *Arabidopsis* (Rock and Zeevaart 1991), *sitiens* and *flacca* of tomato (Taylor 1991), and *aba2* of tobacco (Marin et al. 1996), *vp14* leaves were not prone to wilting. When grown in the green-house where plants were watered daily, the detached leaves of *vp14* mutant seedlings showed a distinctly greater rate of water loss in comparison to wild type siblings and an inbred W22 (Fig. 2-2D). This difference was detected within 5 minutes following leaf detachment, indicating that the stomata in *vp14* leaves did not close as quickly as those of the wild type leaves. However, determination of the bulk levels of ABA in leaves detected no significant difference between *vp14* mutant and wild type siblings (refer to Fig. 2-8B).This apparent contradiction raised a question as to what role changes in the bulk ABA levels have in regulating stomata. It has been previously documented that in stressed leaves stomata close before any increase in bulk ABA can be detected (Walton et

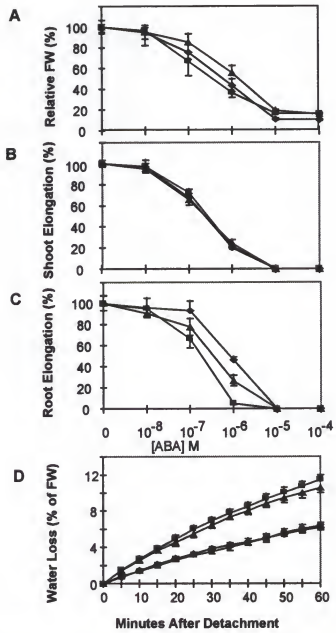


Fig. 2-2. Response of exogenous ABA on germination of embryos cultured *in vitro* (A.B.C), and water loss rates of detached leaves (D) of wild type and *vp14* mutants. Embryos harvested aseptically were placed on MS medium supplied with different concentrations of ABA. Relative fresh weight (A), shoot(B) and root elongation(C) as a percentage of a control (without ABA) were measured after four days. diamond: W22, circle: wild type, square: *vp14-2272*, triangle: *vp14-3250*.

al. 1977). Harris and Outlaw (1991) reported that the increase of ABA in guard cells was faster than changes in other leaf cells when stress was imposed, and more importantly, the guard cell ABA increase correlated with the stomata aperture. Thus, one possible explanation of the water loss phenotype in *vp14* was that it affected a small pool of ABA that regulated stomata aperture. If so, this fast water loss phenomenon may be suppressed in the field grown *vp14* plants by stress induced ABA since ABA can translocate easily.

To test whether vivipary of *vp14* can be inhibited by exogenous ABA, developing embryos were cultured *in vitro* in media containing different concentrations of ABA. Embryos 16 and 18 days after pollination were used because that stage allows fully developed embryos to be isolated before any sign of axis elongation occurs. After 4 days, growth parameters were measured. The *vp14* mutant embryos exhibited the same sensitivity to ABA as did wild type (Fig. 2-2). Precocious germination of *vp14* and wild type embryos was also inhibited by exogenous ABA at the same level, *i.e.* germination was completely inhibited at 10^{-5} M ABA. Embryo growth was inhibited at ABA concentrations greater than 10^{-8} M, consistent with previous reports on other ABA deficient mutants (Robichaud et al. 1986). It was noted that root growth of *vp14* mutant embryos showed an enhanced sensitivity to ABA inhibition compared to the wild type (Fig. 2-2C). The severe allele *vp14-2274* was somewhat more sensitive than the less severe allele *vp14-3250*, in this respect. Together these data suggested that the vivipary of *vp14* is likely attributed to a deficiency in ABA. However, the enhanced ABA sensitivity of the mutant roots is not explained by this conclusion.

In collaboration with Dr. Jan A. D. Zeevaart's lab at Michigan State University, the endogenous levels of ABA in embryos were determined using GC-MS. Embryos of

Table 2-1. ABA levels are reduced in *vp14-2274* embryos.

DAP*	WT sib	*vp14-2274*
	ABA ng/g fr.wt. (%)	
16	83.3 (100)	23.3 (27.8)
18	127.5 (100)	36.1 (28.3)
20	71.0 (100)	43.7 (61.5)

*DAP: days after pollination

16, 18, and 20 days after pollination (DAP) were sampled in order to span the time of ABA regulated gene expression in embryos (McCarty et al. 1991). The *vp14-2274* embryo contained only 28% of the wild type level of ABA at 16 and 18 DAP, and 61% as much as wild type at 20 DAP (Table 2-1). Visible elongation of the embryo shoot axis in the mutant is generally observed at 20 DAP. The significant level of residual ABA may account for the relatively weak penetrance of the *vp14-2274* phenotype. Lower but still significant levels of residual ABA (~10% of wild type) are also evident in embryos of the strongly viviparous carotenoid deficient mutants (e.g. *vp5*) of maize (Neill et al. 1986). The extremely wilty mutant of tobacco *aba2* also retains 23-48% of wild type ABA in leaf tissues (Marin et al. 1996). The origin of this residual ABA is still unknown. ABA levels in other vegetative organs such as leaves and roots appeared to be less affected since no differences were detected in nonstressed root and leaf tissues. The total stress induced ABA synthesis measured after 5 h in detached *vp14-2274* leaves was about 25% lower than the wild type siblings (data not shown). Together these data indicated that *vp14* is an

ABA deficient mutant that predominantly affects the ABA synthesis in developing embryos.

Analysis of ABA Biosynthetic Pathway Intermediates in *vp14* Embryos

ABA is proposed to be synthesized through a sequence of reactions that includesepoxidation of zeaxanthin to violaxanthin, subsequent isomerization of *all-trans* violaxanthin to *9-cis*-violaxanthin, followed by an oxidative cleavage of 9-*cis* xanthophylls to produce xanthoxin which is subsequently converted to ABA (Zeevaart and Creelman 1988, Walton and Li 1995). To locate the blocked step, the C_{40} carotenoids and xanthophyll intermediates in *vp14* mutant embryos were analyzed by HPLC in collaboration with Dr. Zeevaart's lab at MSU. No significant differences in the levels of C_{40} carotenoid precursors were detected between *vp14* and the wild type embryos at 16 or 18 DAP (Fig. 2-3). The identities of those absorption peaks were calibrated either by standard compounds or by published spectral data (Molnar and Szabolcs 1979, Rock and Zeevaart 1991, Parry and Horgan 1992). The mutant embryos have comparable amounts of zeaxanthin, violaxanthin, and slightly increased 9-*cis* violaxanthin and 9-*cis* neoxanthin contents. The blocked step in *vp14* is clearly different than in other viviparous maize mutants such as *vp2*, *vp5*, *vp7* and *vp9* which are depleted in those ABA synthetic precursors (Neill et al. 1986). The *vp14* mutation also differs from the ABA deficient mutant of *Arabidopsis*, *aba1*, or the equivalent mutant of tobacco *aba2*, which are blocked in the epoxidation of zeaxanthin and have markedly lower violaxanthin, 9-*cis* xanthophyll contents (Rock and Zeevaart 1991, Duckham et al. 1991, Marin et al. 1996). The normal or slightly increased 9-*cis* violaxanthin and 9-*cis* neoxanthin levels also

indicated that *vp14* is unlikely to be blocked in the double bond isomerization step. These results suggested that the blocked step in *vp14* is downstream of the 9-*cis* xanthophylls in the pathway. The remaining steps include oxidative cleavage of 9-*cis* xanthophylls to form xanthoxin, xanthoxin conversion to ABA-aldehyde, and oxidation of ABA-aldehyde to ABA.

Table 2-2: Xanthoxin conversion to ABA in *vp14-2274* embryos.

Embryos (20 DAP)	mg protein	ABA(ng)
WT sib	100	18.9+/-2.1
vp14-2274	100	35.0+/-1.4

Many of the known ABA deficient mutants are blocked in the conversion of ABA aldehyde to ABA possibly because this step requires a molybdenum-Fe cofactor, the synthesis of which requires several steps. These mutants include *aba3* of *Arabidopsis* (Leon-Kloosterziel et al. 1996, Schwartz et al. 1997), *flacca*, *sitiens* of tomato (Parry and Horgan 1988), *ckr1*, *iba1* of tobacco (Parry et al. 1991, Bitoun et al. 1990), *nar2a* of barley (Walker-Simmons et al. 1989), and *droopy* of potato (Duckham et al. 1989). A convenient approach to assess the steps downstream of xanthoxin is to incubate xanthoxin with cell free extracts and assay for production of ABA. The ability of *vp14* mutant embryos to convert xanthoxin to ABA was determined in collaboration with Dr. Zeevaart. In this assay, homozygous *vp14-2274* embryos were found to be fully capable of converting xanthoxin to ABA, and did so, surprisingly, at a somewhat rate higher than wild type (Table 2-2). The reason why these enzymes are up-regulated in the mutant

remains to be determined, but these data indicated that *vp14* was not blocked in the steps involved in xanthoxin conversion to ABA. Based on our knowledge of the proposed ABA biosynthetic pathway, the results of enzymatic assay and HPLC analysis of precursors suggested that *vp14* is blocked in the cleavage of 9-*cis* xanthophylls to form xanthoxin.

Theoretically, the suggested *vp14* block would be directly confirmed by the detection of a depletion of the product xanthoxin. However, determination of xanthoxin is experimentally difficult and may not be conclusive in any case because the normal level of xanthoxin in all the plant tissues is reportedly extremely low (Nonhebel and Milborrow 1987, Parry et al. 1988 1990).These data provided a compelling though circumstantial case, that *vp14* is the long-sought mutant blocked in the oxidative cleavage step of the ABA biosynthetic pathway.

Molecular Analysis of *viviparous14*

Because both alleles of *vp14* were isolated from maize strains containing active Robertson's *Mutator* (*Mu*) transposons, it is possible that they were tagged by *Mu* insertions. To clone the *vp14* gene, *vp14* was outcrossed to W22 in order to reduce copy numbers of the *Mu* family elements. Statistically, one outcross to a low *Mu* copy number strain such as inbred W22 is expected to reduce the copy number by 50%. Then a segregating population was created from the outcrosses. Genomic DNA from lines segregating the mutant phenotype upon selfing (Segregating, S) were analyzed by Southern blot hybridization to compare lines that did not segregate (Nonsegregating, NS, *i.e.* homozygous for wild type *Vp14*). An internal *TthI111* fragment of the *Mu1* was used as a probe because it will detect most members of the *Mu* transposon family. Southern

blot hybridization identified a 2.5*kb* XhoI fragment that co-segregates with the mutant (Fig. 2-4A). This fragment was cloned by screening a subgenomic λ-gt10 library constructed using size selected DNA enriched in 2.5 kb fragments. Sequencing of this clone confirmed the presence of a *Mu1* insertion in that fragment. The *Mu1* flanking sequence (~1 kb) was amplified by PCR and used subsequently to isolate overlapping wild type genomic clones and cDNA clones from an embryo cDNA library.

To test whether or not the cloned sequence was from *Vp14*, we analyzed *vp14-3250*, an independent allele that was also isolated from an active *Mu* line. Southern blot analysis of *vp14-2274* and *vp14-3250* DNA showed evidence that each contained a *Mu1* sized polymorphism that was not present in any of six possible progenitor strains that founded the *Robertson's mutator* population (Fig. 2-4B). The corresponding 6.0 and 7.5kb EcoR1 fragments were cloned from *vp14-3250* and subsequent DNA sequencing detected a *Mu1* insertion approximately 1 kb upstream of the *vp14-2274 Mu1* insertion in the 7.5 kb fragment (Fig. 2-4C).

Restriction fragment length polymophorism (RFLP) mapping of *Vp14* probes using recombinant inbred populations (Ben Burr and Frances Burr, pers. comm.: Maize Genome Center, University of Missouri) indicated that *vp14* is located approximately 50 map units proximal to *vp8* on 1*L* in the vicinity of the *bz2* locus. This mapping location was in full agreement with the TB translocation experiment which placed *vp14* on 1L. In addition, a possible duplicate locus was detected and mapped to chromosome 5S. This finding is consistent with a large body of RFLP evidence that 1L and 5S are duplicated

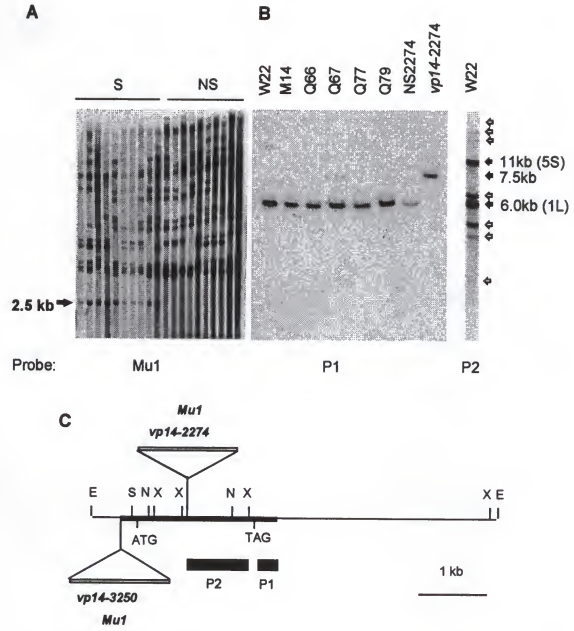


Fig. 2-4. A. Southern analysis of a family segregating *vp14-2274* using a fragment of *Mu1* as a probe. DNA was pooled from several seedlings of each line. The arrow points to a 2.5 kb fragment identified as co-segregating with the *vp14* mutant. B. DNAs of six possible progenitors, *vp14* mutant and wild type plants were hybridized with a *Vp14* specific probe (P1 as indicated in C). On the right, W22 DNA was hybridized by probe2 (P2) at low stringency. The closed arrows point to a 6 kb *vp14* (mapped on 1L) and a putative duplicate (11 kb mapped on 5S). Open arrows point to the closely related fragments. C. The restriction enzyme map of a 6Kb EcoRI fragment containing the *Vp14* gene and the locations of the *Mu1* insertion sites in each allele. The seguences corresponding to two probes used are marked as P1 and P2. E, EcoRI; N, NotI; X, XhoI, Nc, NcoI.

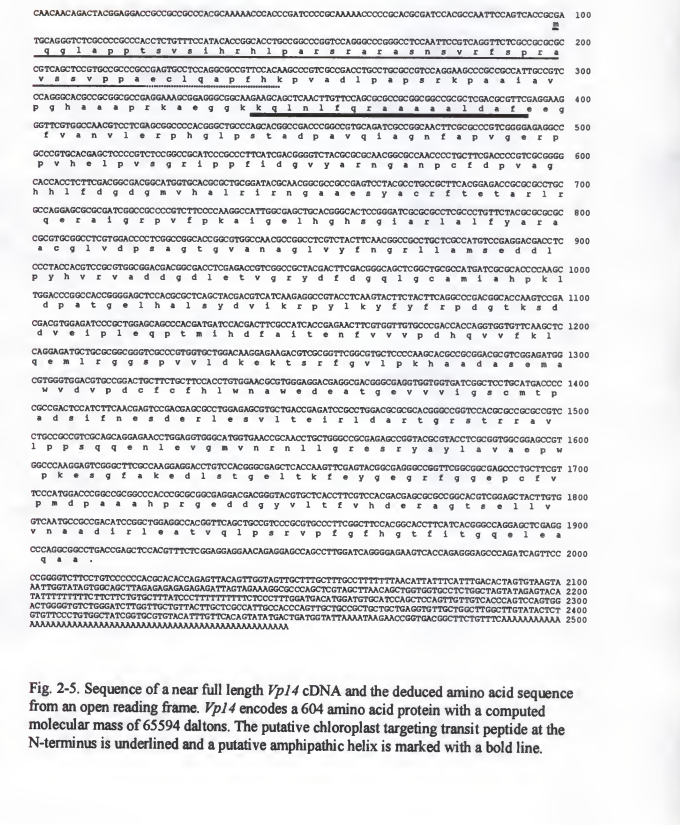


Fig. 2-5. Sequence of a near full length *Vp14* cDNA and the deduced amino acid sequence from an open reading frame. *Vp14* encodes a 604 amino acid protein with a computed molecular mass of 65594 daltons. The putative chloroplast targeting transit peptide at the N-terminus is underlined and a putative amphipathic helix is marked with a bold line.

segments in the maize genome (Helentjaris et al. 1988). Southern blot analysis using the cDNA sequence as a probe suggests that *vp14* belongs to a small gene family. At moderately high stringency, two major EcoRI fragments (6.0 and 11/3.5 *kb*) in inbred W22 genomic DNA were detected corresponding to the 1L and 5S copies respectively. Four to six fragments hybridized at lower stringency (Fig. 2-4B).

Vp14 Is an Intronless Gene

To reveal the structure of the *Vp14* gene, a cDNA library was constructed in λ-ZAP using PolyA enriched RNA isolated from the 18 DAP W22 inbred embryos. Ten nearly full length cDNA clones were recovered from 10^9 primary recombinants when the cDNA library was screened with the 1kb flanking probe (P2). Sequencing of the cDNAs and the 6.0 kb EcoRI genomic clone indicated that *vp14* contains no introns in the transcribed region. A putative TATA box was located in the genomic sequence 80 bp upstream of the longest cDNA. Analysis of the cDNA identified a 1812 bp open reading frame predicted to encode a 604 amino acid protein with a calculated molecular mass of 65.5 kilo-daltons (Fig. 2-5).

VP14 Protein Is Related to a Cleavage Dioxygenase in Bacteria

The BLAST algorithm was used to search for related protein sequences in the non-redundant NCBI protein database. VP14 had a strong similarity to lignostilbene dioxygenase (LSD) of *Pseudomonas paucimobilis* (Kamoda et al. 1993a) and a weaker, but significant similarity to human RPE65, a protein highly specific to the retinal pigment epithelium of the eye (Hamel et al. 1993), and its mammalian homologs. In addition, two related proteins are potentially encoded in the *Synechocytis* cyanobacterial genome. A

multiple sequence alignment of the proteins shown in Fig. 2-6 suggested strongly that the LSD, RPE65 and VP14 are related proteins. The proteins align closely at their C-termini. Several blocks of similar sequence in proteins are clustered around four conserved histidines and one potentially conserved tyrosine residue. In dioxygenases of known structure, conserved histidine and tyrosine residues are typical ligands for the non-heme iron cofactor. It has been confirmed that the VP14 protein expressed in *E. coli* as a GST-fusion protein contains significant amounts of bound non-heme iron (data not shown). LSD also contains non-heme iron (Kamoda et al. 1993a, b). These results strongly reinforce the conclusion that LSD and VP14 define a new class of non heme iron proteins. The VP14 sequence includes an additional 100 amino acid N-terminal extension relative to LSD and RPE65. This N-terminal peptide is consistent with the properties of a chloroplast transit peptide (Cline and Henry 1996) and may serve to target VP14 to plastids where the initial step of ABA synthesis is believed to occur (Zeevaart and Creelman 1988).

LSD catalyses an oxidative cleavage of the central double bond of lignostilbene to form two molecules of the corresponding aldehyde (vanillin) as illustrated in Fig. 2-6. This activity has been confirmed for the recombinant protein expressed in *E. coli* (Kamoda et al. 1993a, b). This oxidative cleavage reaction is chemically analogous to the oxidative cleavage of the double bond in xanthophylls that initiates ABA biosynthesis in plants (Fig. 2-7). These results together with the biochemical characterization, supported the hypothesis that *Vp14* encodes the 9-cis xanthophyll cleavage dioxygenase of the ABA biosynthetic pathway. Of course, still more convincing evidence would be the direct demonstration of the VP14 cleavage activity *in vitro*.

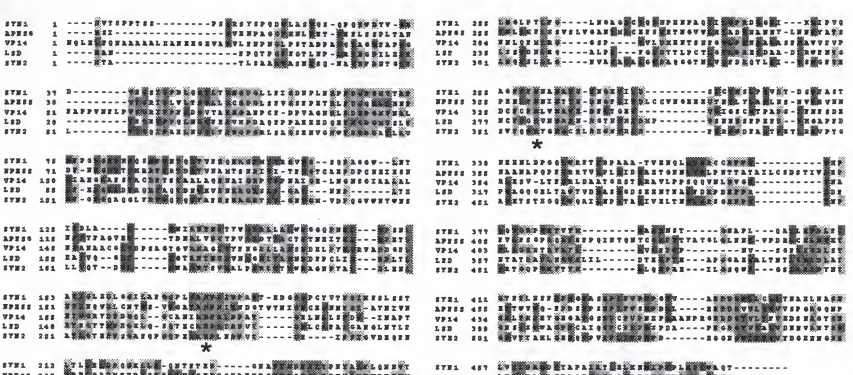
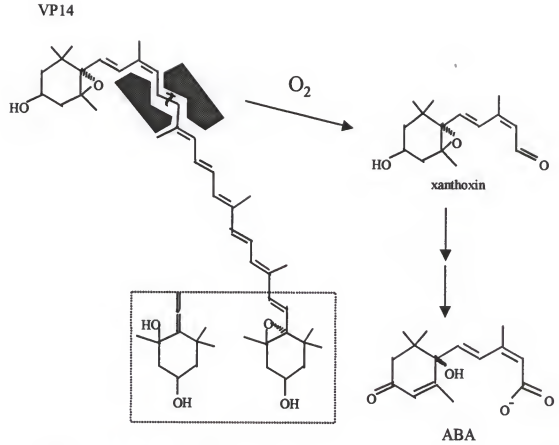


Fig. 2-6. Alignment of VP14 with homologous proteins. LSD: lignostilbene dioxygenase in bacteria. RPE65: a 65 kd protein specific to retinal pigment epithelium cells of bovine. Syn1(D90914, orf) and Syn2 (D90914, orf). Four highly conserved histidine residues were marked with asterisks.



Lignostilbene dioxygenase:

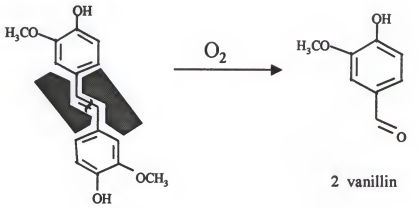


Fig. 2-7. Proposed reactions catalyzed by VP14 and the reaction catalyzed by lignostilbene dioxygenase (LSD) reaction (Kamoda & Saburi, 1993b).

Vp14 Expression Is Developmentally and Environmentally Regulated

The expression of *Vp14* was examined in embryos by northern blot analysis (Fig. 2-7A). A 2.6 kb transcript was detected in the developing embryos of wild type, i.e. W22 and the corresponding non-segregating *Vp14-2274* (*NS-2274*) strain, whereas a series of size altered transcripts were detected in the homozygous *vp14-2274* mutant embryos. The *Vp14* mRNA levels were greatly reduced in the homozygous *vp14-3250* embryos, and a similar pattern of altered sized transcripts was detected. The increased size of the mutant *vp14-2274* and *vp14-3250* transcripts (about 4.0 kb) is consistent with a transcriptional readthrough of the *Mu1* insertion (1.4 kb) in wild type *Vp14* gene (2.6 kb). The smaller sized transcripts in the *vp14* mutant embryos may the aberrantly spliced products due to the *Mu1* insertion. Considering the evidence that *Vp14* belongs to a gene family and the probe may hybridize to homologous sequences, the 4.0 kb transcript in *vp14-2274* was used to identify *vp14* specific RNA in the northern hybridization analysis. In Northern analysis of other tissues, this mutant *vp14-2274* transcript (4.0 kb) was also detected in roots of the mutant seedlings indicating that the *Vp14* gene is normally expressed in roots as well as embryos (Fig. 2-8A). No *Vp14* message was detected in leaf tissues that were grown in normal conditions.

Stress induction of ABA synthesis has been shown in a variety of plant species (Wright and Hiron 1969, Ingram and Bartels 1996), and this induction can be inhibited either by transcriptional or by translational inhibitors (Li and Walton 1990), indicating gene expression is involved in this process. Previous studies have suggested that the cleavage reaction may be the key regulated step in the ABA biosynthetic pathway

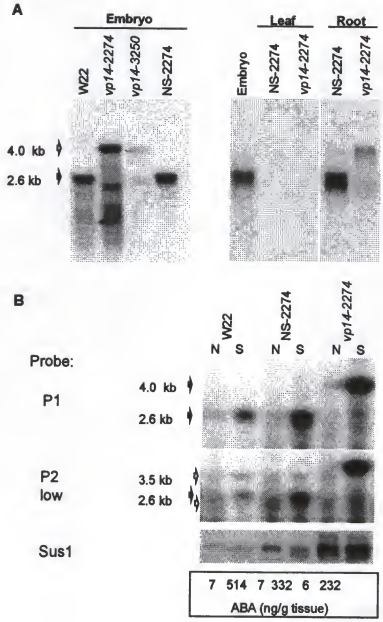


Fig. 2-8. A. Northern analysis using P2 as a probe. The aberrant sized transcripts in *vp14* mutants were marked with open arrows. B. Water stress induction of *Vp14* expression in detached leaves as probed with *Vp14* specific probe (P1) or induction of homologous genes as probed with P2. A hybridization with a *SUS1* (sucrose synthase) probe was provided as control. The ABA levels in those leaves were provided under the lanes.

(Zeevaart and Creelman 1988, Walton and Li 1995). Thus, it was predicted that expression of the key 9-*cis* xanthophyll cleavage enzyme might be stress inducible.

To determine whether *Vp14* expression is environmentally regulated, detached leaves were subjected to a water stress treatment at room temperature for 6 hours. This resulted in a 40 - 70 fold increase of endogenous ABA levels in the *vp14-2274* and wild type leaves (Fig. 2-8B). Two probes were used in the northern blot hybridization of stressed leaves, probe 1 (P1), a 3' untranslated sequence of *Vp14* that is specific to *Vp14* as evidenced by its specific hybridization to *Vp14* transcript (Fig. 2-4B), and broad specificity probe 2 (P2) that can cross hybridize to homologous sequences in W22 genomic Southern (Fig. 2-4B). The *Vp14* message was dehydration inducible as indicated by the strong signal of *vp14-2274* transcript (4.0 kb) in the stressed *vp14-2274* leaves and the strong hybridization of P1 to the 2.6 kb transcript in stressed wild type leaves. Using P2 at low stringency, *Vp14*-related gene expression was detected. A 3.5 kb transcript increases in stressed leaves, and a smaller *Vp14*-related transcript was down regulated upon stress. A probe from the *Sus1* (sucrose synthase) gene was a control for variation in poly(A)-RNA loading. The loading is about equal for nonstressed and stressed samples within each genotype, although it is variable among different genotypes. In any case, these data indicated, as predicted, that expression of *Vp14* and related genes are induced by water stress in plant leaves. This correlates with the stress induced ABA accumulation. However, the *vp14* mutant only partially blocks stress induced ABA synthesis in leaves.

Discussion

Biochemical analysis shows that the *vp14* mutant selectively blocked ABA synthesis in developing embryos and suggests that the *Vp14* gene has a role in developmental control of hormone synthesis in maize. Furthermore, the sequence similarity of VP14 to LSD provided evidence that *vp14* encodes a dioxygenase enzyme responsible for oxidative cleavage of 9-*cis* xanthophylls to xanthoxin.

Variable Dependence of Seed Maturation on ABA in Different Plant Species

ABA is a key regulator of seed maturation and dormancy (Koornneef and Karssen 1994, McCarty 1995, Rock and Quatrano 1995). As documented by ABA deficient mutants (e.g. *vp5*, *vp14*, etc) and the ABA insensitive mutant, *vp1* mutant of maize, a failure to synthesize or to perceive the ABA signal results in a viviparous phenotype. Viviparous seeds bypass the process of maturation, fail to acquire desiccation tolerance, and initiate germination processes while kernels are still attached to the mother plant.

Although some key regulators of the seed maturation pathway such as the VP1 and ABI3 factors are highly conserved between maize and *Arabidopsis*, the dependence of the seed maturation pathway on ABA signaling differs markedly between the two species. In *Arabidopsis*, an ABA dependence of seed maturation is manifest only in mutant backgrounds in which the ABI3 function is attenuated (Koornneef et al. 1994). The mature seed phenotype of the *aba1* mutant shows that the activity of ABI3 is sufficient to result in a normal seed maturation in the absence of ABA. This view is further supported by the fact that even the severe ABA deficient mutants of *Arabidopsis* are able to

complete seed maturation and form desiccation tolerant embryos (Giraudat 1995). In tobacco, the ABA deficient mutant of *aba2* which exhibits severe wilting, also completes the normal seed maturation processes although a reduced dormancy was observed (Marin et al. 1996).

In maize, the dependence of seed maturation on ABA appears to be relatively tight. For the *vp14* mutant, a 70% reduction in bulk ABA levels in embryos during mid embryogeny is evidently sufficient to disrupt seed maturation and thus cause an incompletely penetrant viviparous phenotype. This finding is consistent with the evidence that several strongly penetrant viviparous mutants (e.g. *vp5*) of maize have lower, but still significant levels of residual ABA (~10 % of wild type) in the seed (Neill et al. 1986). Overall it suggests that a threshold level probably greater than 30% of the wild type level ABA is required to complete embryo development *in vivo*. Hence the viviparous, or more precisely the elongated embryo shoot axis phenotype, provides a very sensitive screen for ABA synthetic mutants in maize. This may have contributed to the isolation of *vp14* and other new potentially ABA related mutants in maize (McCarty 1995). Together these results suggest that dependence of the seed maturation pathway on ABA signaling is variable among plant species, and in *Arabidopsis* the default activity of the ABI3 pathway is normally sufficient to complete seed maturation.

Overlapping Sources of ABA Synthesis in Plant Development

The significant levels of residual ABA in embryos and normal levels in vegetative tissues of homozygous *vp14* mutants suggested a possibility of overlapping sources of ABA. Three possible sources may account for the residual ABA in *vp14* embryos: 1) the

existing alleles may be leaky, 2) related genes may be expressed at low levels in the seed, 3) ABA may be transferred from the surrounding maternal tissues that express related genes. Both alleles of *vp14* are caused by a *Mu1* insertion, *vp14-2274* in the central portion of coding region and *vp14-3250* in the 3' untranslated region (Fig. 2-4). It seems unlikely that both alleles of *vp14* are leaky because in order to produce a functional VP14 protein, a perfect slicing of the *Mu1* insertion is likely required from the mutated form of the transcript. Northern blot analysis detected a 4.1 kb transcript that is consistent in size with a *Mu1* insertion in the normal 2.6 kb wild type *Vp14* transcript, and smaller than 2.6 kb sized transcripts that is presumably due to alternative splicing or premature termination resulted from the *Mu1* insertion (Fig. 2-8). A low level of *Vp14* related gene expression in embryos is supported by the recovery of a *Vp14*-like sequence from an embryo cDNA library. Partial sequencing of this clone revealed that the potentially encoded protein is highly homologous to VP14 (data not shown). However, a screen of another cDNA library constructed from mRNA from W22 embryos at 18 DAP did not recover this sequence among the ten *Vp14* clones that were isolated. One possible explanation is that timing of expression of *Vp14* and this *Vp14*-like gene differs during embryo development.

With respect to a third possibility, the normal ABA levels in vegetative tissues of *vp14* mutants and the observation that mutant plants develop normally under field conditions implies strongly that the *Vp14* gene accounts for only a subset of the ABA biosynthetic activity in the plant. Partial suppression of the *vp14* phenotype by ABA transferred from maternal tissues might explain the variable penetrance of the viviparous phenotype we observe under field conditions. Maternal ABA synthesis induced by stress can significantly affect seed development in maize (Ober and Setter 1992). Thus, these

results suggest that embryo ABA may come from at least two sources, synthesis by the embryo itself, which accounts for a majority of the ABA, and ABA translocation from other tissues such as leaves or roots. The endosperm evidently does not contribute significant ABA to the embryos, consistent with the earlier conclusions of Robertson (1952) and Neill et al. (1983). Using T-B translocations in maize, they created kernels with embryos homozygous for *vp5*, but wild type endosperm. The phenotype of these embryos were still viviparous and ABA deficient.

Several lines of data provided indirect evidence that functionally VP14 equivalent genes are expressed in vegetative tissues of the plant. On moderate stringency Southern blots, the 1 kb *Vp14* probe (*P2*) hybridized to nine EcoRI fragments of inbred W22 DNA (Fig. 2-4A). Sequencing of a *Vp14* duplicated sequence (the 11 kb EcoRI fragment in Fig. 2-4A) indicated that it may potentially encode a protein with similar function to VP14 (see chapter 5). The northern blot analysis of leaves also detected expression of related genes (Fig. 2-8B). In addition, we have isolated several distinct, but related cDNAs from root and leaf libraries (B. C. Tan and D. R. McCarty, unpublished data). The normal levels of endogenous ABA in leaves and roots clearly indicate that ABA is synthesized in vegetative tissues of the mutant. And the activity of ABA synthesis is only slightly affected in stressed *vp14 –2274* leaves since ABA can still rise about 40 fold, although not as much as in wild type (Fig. 2-8B). Data base searches identified several *Vp14* related sequences in the rice, maize and *Arabidopsis* EST collections. For these reasons, we suggest that *Vp14* belongs to a small family of differentially regulated genes that contribute to developmental control of ABA biosynthesis in plants.

Role of *Vp14* in Regulation of Stomata in Leaves and in Root Development

Many of the characterized ABA deficient mutants display a wilty plant phenotype, indicating that regulation of stomata closure is affected in these mutants (refer to Chapter 1 for details). Field grown *vp14* mutants did not show any discernible plant phenotype in addition to the seed vivipary. However, detached leaves of the *vp14* mutant seedlings grown in the greenhouse with abundant water supply show enhanced rates of water loss. This feature suggested that stomatal closure is affected by the *vp14* mutation. Measurement of the ABA levels in leaves of these plants detected no significant difference between *vp14* mutants and the wild type siblings, suggesting that the bulk pool of ABA is unchanged. Northern blot analysis has confirmed that in addition to embryo expression, *Vp14* is also expressed in roots and is stress inducible in leaves (Fig. 2-8), indicating the potential involvement of *Vp14* in the ABA synthetic activity in these tissues. However, *Vp14* may only account for a small set of the ABA detected in those tissues based on these results. In many plant species, bulk ABA level increases dramatically in stressed leaves (reviewed by Ingram and Bartels 1996). However, when the time course is taken into account, the closing of stomata generally occurs before any significant changes in ABA content can be detected in the leaves. One possible explanation for this time discrepancy may involve a rapidly localized accumulation of ABA in or around guard cells, either through re-distribution of ABA among leaf cells or through highly localized *de novo* synthesis of ABA, *e.g.* only in guard cells or the neighboring cells. In support to this suggestion, a rapid increase in the ABA concentration of a single guard cell is correlated with the closure of stomata in stressed leaves (Harris and Outlaw 1991). However, this study did not identify the sources of the ABA. Thus, the role of *Vp14* in regulating

stomata closure may be explained by the existence of a highly localized pool of ABA in leaves which specifically affects stomatal closure. ABA in this pool may result from re-mobilization of ABA from other sites such as roots, or from a highly localized expression of *Vp14*, in a limited number of cells, which may not be detected by northern blot analysis. *Vp14* is expressed in normal roots which may be a significant source of ABA in leaves that regulates stomatal aperture (Fig. 2-8). In several species, variations in stomatal conductance are well correlated with the ABA present in xylem, and the initial stomatal close occurs before the shortage of soil moisture causes any measurable change in the water status of the leaves (Zhang and Davies 1989).

Another feature of germinating *vp14* embryos is that their roots show a slightly enhanced sensitivity to exogenous ABA compared to wild type. ABA is required for root elongation in stressed conditions based on evidence that the ABA deficient *vp5* mutant showed poor root elongation compared to wild type (Sharp et al. 1994). The role of ABA in root development and adaptation to environments is less understood. However, the root phenotype of *vp14* may be explained by an increasing hormone sensitivity to compensate for the reduced ABA synthesis.

Evidence that VP14 Is the 9-*cis* Xanthophyll Cleavage Dioxygenase

Deficiency of ABA in the *vp14* embryos indicates that ABA biosynthesis is blocked (Table 2-1). The existence of normal or slightly higher than normal levels of 9-*cis* violaxanthin and 9-*cis* neoxanthin in *vp14* embryos suggests that steps leading to synthesis of these two ABA precursors are not affected. In this respect, *vp14* is clearly different from two related ABA deficient mutants, *aba1* of *Arabidopsis* and *aba2* of tobacco, which

accumulate zeaxanthin and deplete violaxanthin due to a block in the epoxidation step (Duckham et al. 1991, Marin et al. 1996, Rock and Zeevaart 1991). A significant increase of 9-*cis* xanthophylls does not occur in *vp14* embryos, possibly because ABA concentration is normally much lower and only accounts for a small fraction of xanthophyll synthesis (Parry and Horgan 1992). The fact that cell free extract from the *vp14* embryos can convert xanthoxin to ABA ruled out the possibility that *vp14* is blocked downstream of xanthoxin. Thus, by elimination, these data suggest that the blocked step in *vp14* is the oxidative cleavage of 9-*cis* xanthophylls to yield xanthoxin.

Two other possible approaches may provide further evidence for this conclusion: 1) confirmation that xanthoxin pools are depleted in the mutant, 2) direct measurement of the enzymatic activity converting 9-*cis* xanthophylls to xanthoxin, *in vitro*. However, this has proven technically difficult. Firstly, it was reported that xanthoxin levels are extremely low in plant tissues, which in turn provided the evidence that downstream enzymatic activities in converting xanthoxin to ABA may be constitutively active (Sindhu et al. 1987, Li and Walton 1990). Experimentally, direct measurement of xanthoxin in developing embryos is not feasible. With respect to the second approach, an *in vitro* assay of the xanthophyll cleavage activity has not been developed. *In vitro* assays of the carotenoid cleavage activity may be hindered by the extremely low abundance of the enzyme, insolubility of the substrates, and complications arising from nonspecific cleavage reactions by enzymes such as lipoxygenases (Creelman et al. 1992, Schwartz et al. 1997). In fact, it is these difficulties and complications that have greatly hindered previous progress in the search and demonstration of such a dioxygenase in the ABA biosynthetic pathway. The incomplete characterization of the *notabilis* mutant of tomato (Parry and

Horgan 1992, Taylor 1991), has been due to a combination of its leaky nature and the lack of more precise, direct approaches.

Molecular cloning of *Vp14* provides an independent line of evidence at the molecular level. VP14 showed high similarity to a bacterial enzyme, lignostilbene dioxygenase (LSD) (Kamoda et al. 1993). Based on the proposed ABA biosynthetic pathway, cleavage of 9-*cis* xanthophylls was predicted to involve a dioxygenase type enzyme (Walton and Li 1995, Creelman et al. 1992), which breaks the conjugated double 11,12 bond and produces xanthoxin and a C_{25} apo-aldehyde, each with an aldehyde group. No such dioxygenases have been reported in the plant or animal kingdoms (Prescott and John 1996). The reaction catalyzed by LSD is highly analogous chemically to the proposed oxidative cleavage of 9-*cis* xanthophylls. Indeed, the bacterial LSD is the only known enzyme capable of catalyzing the specific oxidative cleavage of a conjugated double bond to form two aldehyde products and would, in the absence of other evidence, provide a compelling model for the carotenoid cleavage enzyme (Fig. 2-7). Lipoxygenases can also generate a series of aldehyde cleavage products of carotenoids *in vitro*, but do so nonspecifically (Creelman et al. 1992, Schwartz et al. 1997). These results, together with biochemical analyses of the *vp14* mutant, build a strong though circumstantial case that VP14 encodes the cleavage dioxygenase in ABA synthesis. Recent analyses of the purified recombinant VP14 have confirmed that it catalyzes oxidative cleavage of 9-*cis* violaxanthin to xanthoxin *in vitro* (next chapter).

Chapter 3

Viviparous14 Encodes a 9'-*cis* Neoxanthin/9-*cis* Violaxanthin Dioxygenase of Abscisic Acid Biosynthesis in Maize

Introduction

Abscisic acid (ABA), a sesquiterpene plant hormone, is involved in regulation of many physiological processes throughout plant development, including germination, transpiration (stomata aperture and root conductivity), seed maturation, dormancy and tolerance to environmental stresses (Zeevaart and Creelman 1988, Walton and Li 1995, Giraudat 1995, Ingram and Bartels 1996). ABA is a particularly interesting hormone with regard to its regulation. The levels of ABA can rise or fall about two magnitudes within 4 to 8 hours in response to environmental and developmental changes (Walton and Li 1995). In fungi, ABA is synthesized from C_{15} intermediates (farnesyl pyrophosphate) *via* a so called "direct pathway"(Zeevaart and Creelman 1988). While in higher plants, ABA is proposed to be synthesized from carotenoids. Blockage of carotenoid biosynthesis in several viviparous mutants of maize (*vp2*, *vp5*, *vp7* and *vp9*) also prevents ABA biosynthesis (Neill et al. 1986, Taylor 1991). Incorporation of ^{18}O from O_2 in the -CHO group instead of the ring of ABA strongly suggested oxidative cleavage of epoxy-carotenoids as the origin of ABA as opposed to direct synthesis from a C_{15} intermediate (Zeevaart et al. 1989). Biochemical analysis of stressed leaves indicated 9-*cis* violaxanthin or/and 9-*cis* neoxanthin are probable substrates that are cleaved to produce xanthoxin (Li and Walton 1990, Parry et al. 1990). Xanthoxin is subsequently converted to ABA

(Zeevaart and Creelman 1988, Walton and Li 1995). In support of this pathway, a number of mutants that are blocked at several of the individual steps of the pathway have been isolated (reviewed by Taylor 1991, Leon-Kloosterziel et al. 1996, Schwartz et al. 1997, Marin et al. 1996). Many of the characterized ABA specific mutants are blocked in the conversion of ABA aldehyde to ABA (Taylor 1991). An ABA deficient mutant of *Arabidopsis*, *aba1*, is shown to be blocked in the epoxidation of zeaxanthin (Duckham et al. 1991, Rock and Zeevaart 1991). A zeaxanthin epoxidase was recently cloned in tobacco and was shown to complement the *aba1* mutant in transgenic *Arabidopsis*. However, direct evidence for an enzymatic activity with the proposed specificity for epoxy-carotenoid cleavage in ABA biosynthesis has not yet been found due to difficulties arising from low abundance and liability of the enzyme (Walton and Li 1995, Schwartz et al. 1997). Development of an *in vitro* assay has been hindered by the presence of lipoxygenases and peroxidases, which oxidatively cleave carotenoids in a non-specific manner (Creelman et al. 1992).

Biochemical characterization of a seed specific ABA deficient mutant of maize, *viviparous14* (*vp14*), indicates that it is blocked in the cleavage of epoxy-carotenoids in the ABA biosynthetic pathway (Tan et al. 1997, Chapter 2). The existence of normal or perhaps slightly higher levels of 9-*cis* neoxanthin and 9-*cis* violaxanthin in mutant embryos indicates that *vp14* is not blocked in steps prior to the formation of 9-*cis* carotenoids. The extracts of the mutant embryos are fully active for enzymatic activities required for conversion of xanthoxin to ABA. Together, these results suggested that *vp14* is impaired in the oxidative cleavage step of epoxy-carotenoids in ABA biosynthesis. Experimental results described in the previous chapter show that VP14 is related to a bacterial enzyme

lignostilbene dioxygenase (LSD) (Kamoda and Saburi 1993a, b) which catalyzes a reaction that is strikingly analogous to the oxidative cleavage of 9-*cis* epoxy carotenoids in the ABA biosynthetic pathway in plants. In this chapter, purified recombinant VP14 as expressed in *E. coli* is shown to specifically cleave 9'-*cis* neoxanthin and 9-*cis* violaxanthin to produce the ABA precursor xanthoxin, and a corresponding C_{25} apo-aldehyde. Consistent with other dioxygenases (Prescott and John 1996), recombinant VP14 contains a non-heme iron which is critical to its activity.

Material and Methods

Expression of VP14 Recombinant Protein A fragment containing the coding region and 3' untranslated sequences of the *Vp14* cDNA was amplified by polymerase chain reaction (PCR) using a forward primer (5'-ATGCGGATCCATGCAGGGTCTCG-CCCCG) and a T7 reverse primer. A BamHI site was engineered in the 5 primer very close to the first ATG to facilitate cloning. The amplified fragment was ligated into the BamHI and EcoRI sites of pGex-2T expression vector to express a GST-VP14 fusion protein in *E. coli*, JM109. Synthesis of the fusion protein was induced by adding 0.1 mM IPTG to mid-log phase culture grown at 32 °C in 2xYT-G medium supplemented with 0.4 mM Ferrous iron. GST-VP14 was purified by binding to Glutathione Sepharose 4B and VP14 was isolated by digestion with thrombin according to the manufacture's instruction (Pharmacia). Purified VP14 was quick frozen in aliquots in liquid nitrogen and shipped on dry ice for enzymatic assay at Michigan State University.

Determination of Iron The iron content of the purified VP14 protein was determined by a colorimetric method described by Perciva et al.(1991). 30%

trichloroacetic acid was added to the protein solution to a final concentration of 5% to release the non-heme iron. After centrifugation to remove the precipitate, the 105 µl supernatant was transferred to a new tube and 20 µl saturated ammonium acetate, 12.5 µl ascorbate (0.12M) and 12.5 µl FerroZine (0.25M) reagent were added. After incubation at room temperature for 30 minutes, absorbance at 562 nm was determined. The protein concentration was measured by Bradford method using BSA (bovine serum albumin) as of standard (Bradford 1976) using bovine serum albumin (BSA) as a standard..

Preparation of the Substrates The substrates used for VP14 enzymatic assay test were isolated by Steve Schwartz and Jan Zeevaart at Michigan State University as described (Schwartz et al. 1997). Briefly, crude thylakoid membranes were isolated from spinach leaves. The carotenoids were saponified and partitioned to ether and then resolved on a semiprep µPorasil HPLC column (Waters). Fractions were collected and the identities of prepared substrates were confirmed by spectrum analysis in comparison with standards or published data (Molnar and Szabolcs 1979, Parry and Horgan 1991). 9-*cis* zeaxanthin was prepared by iodine isomerization of the *all-trans* zeaxanthin (Zechmeister 1962).

Enzymatic Activity of Recombinant VP14 Protein The enzyme assays were performed by Schwartz and Zeevaart. About 6 µg VP14 was incubated with each substrate in a reaction buffer containing 100 mM BisTris, pH 6.7, 0.05% Triton X-100, 10 mM ascorbate, 5 µM $FeSO_4$ and 1 mg/ml catalase. Assays were performed at room temperature under red light to minimize photo-oxidation of the precursors and product. Purified 9-*cis* neoxanthin was initially used to test for cleavage. The products were analyzed by NP-HPLC and they were C_{25} apo-aldehyde fragment and xanthoxin. For

determination of substrate specificity, VP14 was incubated with purified *all-trans/9-cis* neoxanthin, *all-trans/9-cis* violaxanthin and *all-trans* zeaxanthin / 9-*cis* zeaxanthin suspended in Triton X-100 and ascorbate. After the reaction, the products were resolved on a TLC plate and stained for aldehyde with 2,4-dinitrophenylhydrazine.

Mass-Spectrometry Analysis of Reaction Products As described in detail by Schwartz et al. (1997), the identity of xanthoxin was confirmed by GC-MS of the TMSi derivative according to Gaskin and MacMillan (1991). The C_{25} apo-aldehydes were analyzed by electron impact mass spectrometry with direct inlet sampling by Dr. Douglas Gage at the mass spectrometry facility at Michigan State University. The expected fragmentation pattern of both C_{25} product was derived from published spectra (Molnar and Szabolcs 1979, Parry and Horgan 1991).

Results

Recombinant VP14 Protein Contains Non-heme Iron

Biochemical characterization and molecular analysis have provided compelling evidence indicating that *Vp14* encodes the 9-*cis* epoxy-carotenoid cleavage dioxygenase (Chapter 2, Tan, et al. 1997). To directly test the proposed activity of VP14, the coding region of the *Vp14 cDNA* was fused to a glutathion-S-transferase (GST) gene and expressed in *E. coli* (Fig. 3-1A). SDS-PAGE analysis of the affinity purified proteins detected a 90 kd fusion protein (GST-VP14) that was induced by IPTG. Cleavage of that fusion protein by the site specific proteinase thrombin (LVPR↓GS) produced two major proteins, the GST domain and the VP14 recombinant protein which presumably carried two additional amino acids (Gly-Ser-) at its amino terminus. Binding of GST to

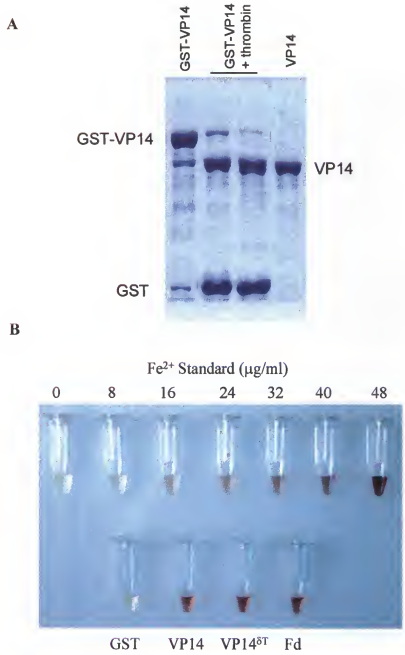


Fig. 3-1. Expression and purification of recombinant VP14 from *E. coli* (A) and determination of iron in the protein (B). The recombinant VP14 has extra Gly-Ser- at its N-terminus; $VP14^{\delta T}$ bears a 30 amino acid truncation in the putative transit peptide at N-terminus. Fd stands for ferrodoxin.

glutathione sepharose 4B facilitated an easy purification of recombinant VP14. Because proper folding of VP14 may require the presence of ferrous iron, the *E. coli* cultures were supplied with 0.4 mM iron. The purified protein was analyzed for iron using the cleavage produced GST protein as a control. As shown in Fig. 3-1B, purified recombinant VP14 contains significant amounts of iron. Accurate determination of iron ratio in prepared VP14 by FAB-MS in collaboration with Dr. B. W. Smith (Department of Chemistry, University of Florida) revealed a stoichiometry about 0.3, which hints that either some protein lost their iron or the active form of the protein is an oligomer. Later assays which showed that addition of ferrous iron proportionally increased the activity of VP14 favored the first possibility.

VP14 Cleaves 9-*cis* Neoxanthin into Xanthoxin and a C_{25} Product

In collaboration with Steve Schwartz and Jan Zeevaart (Michigan State University), the recombinant VP14 protein was tested for cleavage activity using 9-*cis* neoxanthin as a substrate. The cleaved products were analyzed by HPLC and thin layer chromatograph (TLC). The expected cleavage products, xanthoxin and the C_{25} allenic apo-aldehyde, were identified by their UV/VIS absorption spectra and by mass spectra obtained by Dr. Douglas Gage at the mass spectrometry facility at Michigan State University (Schwartz et al. *1997*). The fragmentation patterns for the epoxy- and allenic-C_{25} cleavage products were nearly identical to published spectra for these compounds (Molnar and Szabolcs 1979, Parry and Horgan 1991). Exact mass measurements described in Schwartz et al. 1997 match the theoretical mass of the isomeric C_{25} cleavage products from neoxanthin and violaxanthin ($C_{25}H_{34}O_3$) is 382.2508. The measured mass of

the C_{25} product from neoxanthin was 382.2498 with an error of -2.6 ppm. The measured mass of the C_{25} product from violaxanthin was 382.2501 with an error of-1.8 ppm. Thus, the cleavage is at the 11,12 double bond of 9-cis violaxanthin and 9-cis neoxanthin to produce one molecule of xanthoxin (C_{15}) and one molecule of C_{25} apoaldehyde (allenic and epoxy apoaldehyde). This cleavage of 9-*cis* epoxy-carotenoids by VP14 is well matched with the proposed reaction in the ABA biosynthetic pathway. Xanthoxin was detected with the absorbance at 280 nm, the 9-*cis* neoxanthin and the C_{25} cleavage product were detected at 412 nm. As shown in Fig. 3-2, VP14 cleaves 9-*cis* neoxanthin to produce two products, xanthoxin (C_{15}) and a C_{25} apoaldehyde which was identified by mass-spectrometry to be C_{25} allenic apo-aldehyde with molecular mass of 382.2498 with an error of –2.6 ppm. Quantification of the products revealed an equimolar ratio of xanthoxin and the C_{25} product, consistent with a specific cleavage at the 11-12 double bond of the polyene chain. Non-enzymatic cleavage resulting from photo-oxidation or Fenton reaction would result in random cleavage at different double bond positions (Schwartz et al. 1997).

A number of factors were tested to optimize the VP14 cleavage activity and the results indicated that molecular oxygen (co-substrate), ferrous iron, and a detergent were all necessary for the *in vitro* cleavage activity (Schwartz et al. 1997). According to the $^{18}O_2$ incorporation studies in stressed leaves (Creelman and Zeevaart 1984, Creelman et al 1987, Li and Walton 1987, Parry et al. 1988, Zeevaart et al. 1989), the molecular oxygen is the co-substrate and each atom of O_2 is incorporated into the -CHO group of the reaction products, xanthoxin and C_{25} apo-aldehyde (Parry and Horgan 1991). Depletion of O_2 in the reaction completely abolished the cleavage activity. A supplement

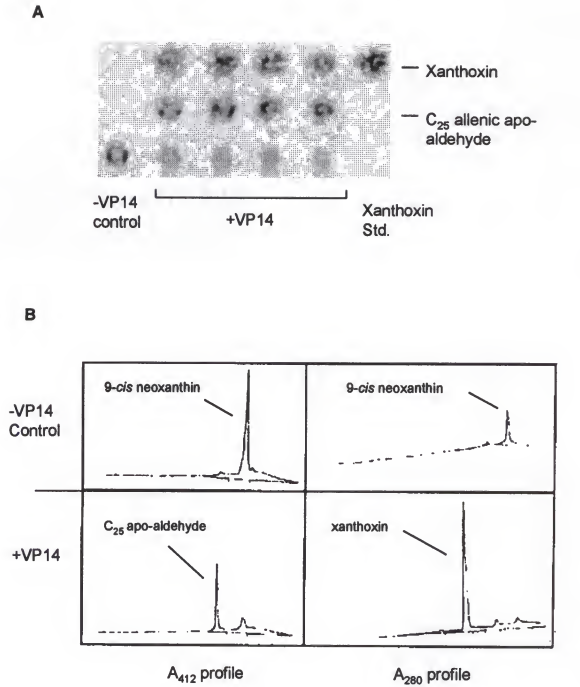


Fig. 3-2. TLC analysis (A) and NP-HPLCanalysis (B) of the reaction products after 9-*cis* neoxanthin was incubated with recombinant VP14 purified from *E. coli*. The TLC plate was stained by 2,4-dinitrophenylhydrazine to detect aldehydes. The test was performed in collaboration with Steve Schwartz and Jan Zeevaart.

of ferrous iron, but not ferric iron, in the reaction mixture resulted in an about 3 fold increase in the cleavage activity. Addition of divalent cation chelator EDTA led to a complete inhibition of the cleavage activity, while removal of EDTA and addition of Fe2+ sufficiently restored the activity. These results support the idea that ferrous iron is a critical co-factor of this epoxy-carotenoid dioxygenase. This is consistent with the substoichiometric amount of iron detected in the purified recombinant VP14 protein. The requirement for Triton X-100 in the assay is probably due to its role in solubilizing the substrate. It may not reflect the *in vivo* reaction environment since carotenoids are shown to exist in association with plastid membranes (Bartley and Scolnik 1995, Markwell et al. 1992). A slight increase in xanthoxin production by adding catalase is possibly caused by reducing the nonenzymatic degradation of the substrate, *e.g.* by a Fenton reaction (Schwartz et al. 1997). Ascorbate was added to the reaction to maintain Fe^{2+} in the reduced state.

9-*cis* Configuration Defines the Substrate Specificity of VP14

Further studies of recombinant VP14 performed at Michigan State University determined the substrate specificity of the cleavage reaction. The reaction products from various carotenoid substrates were separated on TLC plates and stained with 2,4-dinitrophenyl hydrazine to detect aldehyde as shown in Fig. 3-3. The *all trans* isomers of violaxanthin and neoxanthin were not cleaved by VP14. Xanthoxin and the predicted C_{25} products are present only in reactions containing the *9-cis* violaxanthin and 9-*cis* neoxanthin as indicated by the xanthoxin standard.

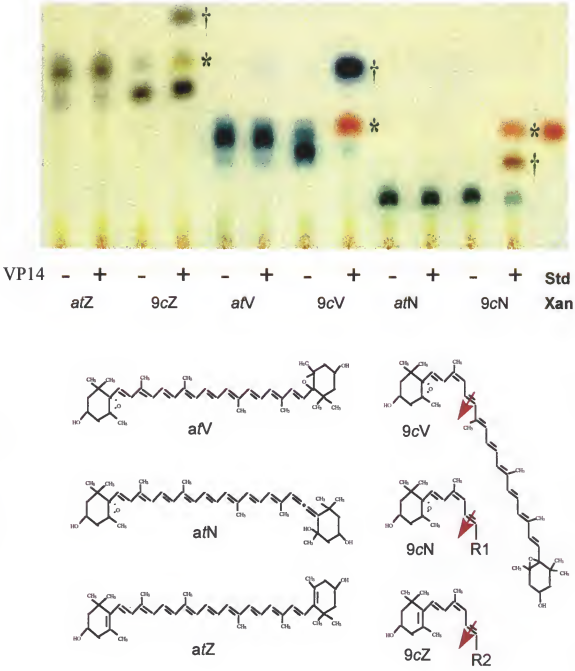


Fig. 3-3. Staining of a TLC plate that resolved the reaction products of specific substrate incubated with recombinant VP14 (Top, adapted from Schwartz et al, 1997). The structure of the tested substrates were shown below. *at*V= *all trans* violaxanthin, *at*N= *all trans* neoxanthin *at*Z= *all trans* neoxanthin, and 9*c*= 9-*cis* isomers. R1 and R2 represented the corresponding residue in *all trans* configuration.

9-*cis* zeaxanthin was also cleaved, presumably yielding a C_{15} and a C_{25} apoaldehyde which suggested that the 9-*cis* configuration is a primary determinant of VP14 substrate specificity. Cleavage of 9-*cis* zeaxanthin does not produce xanthoxin, and this reaction is not known in plants possibly because that zeaxanthin exists only in the *all trans* configuration. The *9-cis* zeaxanthin used in above reaction was formed by iodine isomerization of the *all-trans* zeaxanthin (Schwartz et al. 1997). Other mono-epoxy carotenoids, antheraxanthin and lutein epoxide also exist in the *all-trans* configuration (Parry et al. 1990) and, as expected, their *all-trans* isomers were not cleaved by VP14 (Schwartz, Tan, Zeevaart and McCarty, unpublished data). Thus, *Vp14* encodes the 9-*cis* neoxanthin/9-*cis* violaxanthin dioxygenase of the ABA biosynthetic pathway in maize, and this specific carotenoid cleavage activity has been directly demonstrated *in vitro*.

Discussion

Cloning and expression of VP14 reinforced the conclusion made by physiological and biochemical characterization of the mutant that *vp14* is blocked in the oxidative cleavage of oxygenated carotenoids. Furthermore, the activity of VP14 provided convincing evidence to the identity of the *Vp14* clone. VP14 as expressed in *E. coli* can specifically cleave the 11, 12 double bond of 9-*cis* oxygenated carotenoids into a C_{25} and a C_{15} product, each with an aldehyde group (Fig. 3-3). Xanthoxin, the precursor of subsequent ABA biosynthetic reactions, is formed only by cleavage of 9-*cis* violaxanthin and 9'-*cis* neoxanthin. And one molecule of 9'-*cis* neoxanthin or 9-*cis* violaxanthin produces one molecule of xanthoxin and one molecule of C_{25} apoaldehyde. Thus, it is in good agreement with the proposed reactions of an oxidative cleavage of 9-*cis*

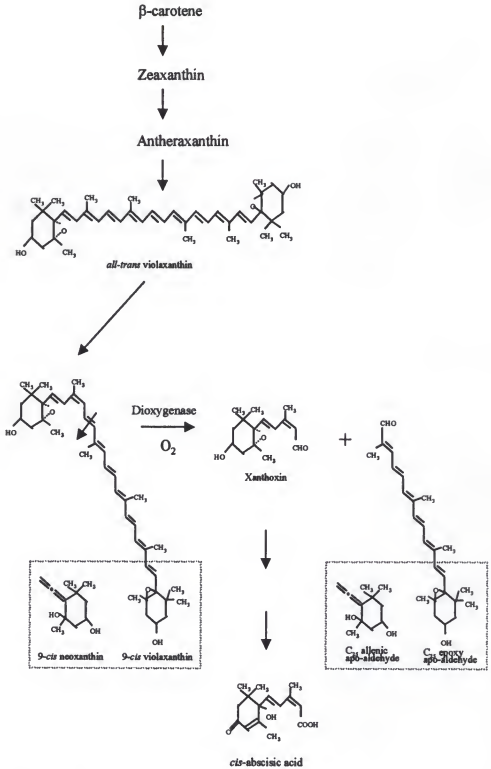


Fig. 3-4. The ABA biosynthetic pathway in higher plants and the function of VP14 as a dioxygenasae. The cleaved double bond by VP14 was marked by a arrow. 9'-*cis* neoxanthin and 9-*cis* violaxanthin are the substrates to produce xanthoxin and only different residues were shown in the boxes.

xanthophylls in the ABA biosynthetic pathway in plants as shown in Fig. 3-4 (Zeevaart and Creelman 1988, Walton and Li 1995). The fate of the C_{25} fragments in *vivo* is not known. The affinity of VP14 for 9'-*cis* neoxanthin and 9-*cis* violaxanthin was not determined. Because the former is more abundant than the latter in a number of species including maize, tomato, bean and spinach (Li and Walton 1990, Parry and Horgan 1992), 9'-*cis* neoxanthin might be the major substrate of VP14. The decrease of 9'-*cis* neoxanthin in stressed leaves of a number of species accounted for most of the increase of ABA (Li and Walton 1987, 1990, Parry et al. 1990, Sindhu et al. 1988).

VP14 is also able to cleave 9-*cis* zeaxanthin, which lacks the epoxide on the ring compared to the normal substrates, suggesting that the 9-*cis* configuration is a primary factor that determines the substrate specificity. It is reasonable to speculate that both the ionone ring and the distance between the 9-*cis* double bond and ring structure also determine the substrate specificity. Inferred from that, all of the 9-*cis* isomers such as 9-*cis* lutein, 9-*cis* antheraxanthin and 9-*cis* zeaxanthin may potentially serve as the substrates of VP14. However, the 9-*cis* isomers of these carotenoids are not found in plants (Parry et al. 1991). The 9-*cis* zeaxanthin used for cleavage reaction was made by iodine isomerization of the *all-trans*-zeaxanthin *in vitro* (Zechmeister 1962). The predominant *cis* xanthophyll in either light or dark grown bean leaves was found to be 9-*cis* neoxanthin, and 9-*cis* violaxanthin only accounted for a small percentage (Li and Walton 1990). What role, if any, light plays in the isomerization of *all-trans* carotenoids to generate 9-*cis* isomers needs to be answered, however, roots grown in darkness normally contain 9-*cis* carotenoids. The specific abundance of 9-*cis* violaxanthin and 9-*cis* neoxanthin in dark grown leaves suggests involvement of a specific isomerase in the *trans* to *cis* conversion.

The rapid and specific increase of 9-*cis* violaxanthin and 9-*cis* neoxanthin in stressed dark grown bean leaves is consistent with that hypothesis (Li and Walton 1990).

The lignostilbene dioxygenases from *Pseudomonas* and VP14 comprise a novel class of dioxygenases that catalyze similar double bond cleavage reactions and generate products with an aldehyde group. The conserved sequences have also been identified two ORFs in the complete genomic sequence of the cyanobacterium *Synechocystis.* Functions of the gene products have not yet been determined. However, related cyanobacteria are known to synthesize at least two apo-carotenoids from β-carotene, cyclocitral and β-ionone (Fresnedo et al. 1991). The demonstration of VP14' role in cleaving carotenoids may clarify vitamin A biosynthesis in animals as it was proposed that a similar cleavage reaction of β-carotene to generates vitamin A. This 15, 15-dioxygenase has been controversial (Yeum 1995, Wolf and Phil 1995). Thus, conserved sequences may be useful in identifying such carotenoid cleavage dioxygenases in the animal intestine.

The significant similarity of VP14 to RPE65 of the human retinal pigment epithelium is intriguing in the light of recent evidence that REP65 may catalyze an isomerization of *all trans* retinol to 11-*cis* retinol (Micheal Redmond, NIH, unpublished data). RPE65 was cloned and found to be expressed specifically in the retinal pigment epithelium cells (Hamel et al. 1993). VP14 does not have any detectable isomerase activity as indicated by testing *all-trans* substrates. However, some of the VP14 homologous genes may encode the isomerase as a large number of *vp14* related genes in plants are detected by DNA hybridization and database searches. (Fig. 2-4, chapter 2). It has been suggested that 9-*cis* isomerase, if exists, should also be stress inducible as synthesis of 9-*cis* violaxanthin and neoxanthin is promoted upon stress (Li and Walton

1990). Further study of those homologous sequences may possibly resolve these questions.

Chapter 4
Localization of VP14, a *9-cis* Violaxanthin/*9-cis* Neoxanthin Dioxygenase Involved in ABA Biosynthesis

Introduction

Abscisic acid (ABA) is involved in the regulation of various plant processes including seed maturation, dormancy and plant responses to a variety of stress conditions (Ingram and Bartels 1996, McCarty 1995, Pena-Cortes and Willmitzer 1995, Rock and Quatrano 1995). ABA is an apo-carotenoid derived from oxidative cleavage of *9-cis* epoxy carotenoids, a step that is widely believed to be the key regulated step in ABA synthesis (Creelman et al. 1992, Zeevaart and Creelman 1988), whereas the enzymes required for conversion of xanthoxin to ABA appear to be constitutively active in most plant tissues (Sindhu and Walton 1987). Thus, xanthoxin does not accumulate to significant levels in normal or water stressed plant tissues.

In maize, the cleavage dioxygenase was cloned by transposon tagging from an ABA deficient *viviparous14* mutant that is blocked in that step (Tan et al. 1997, Refer to Chapter 2). VP14 recombinant protein expressed in *E. coli* was shown to cleave specifically *9-cis* violaxanthin and *9-cis* neoxanthin to produce xanthoxin, an immediate precursor for ABA synthesis (Schwartz et al. 1997). ABA biosynthetic enzymes that utilize carotenoids as substrates are believed to be localized in plastids since carotenoids exist exclusively in plastid membranes. In tobacco, the epoxidase which converts zeaxanthin to *all trans*-violaxanthin was found targeted to chloroplasts (Marin et al.

1996). It appears that the cleavage product, xanthoxin, can be converted to ABA in the cytosolic extraction, indicating the subsequent enzymes may be cytosolically localized (Sindhu and Walton 1988, Walton and Li 1995). The substrates for VP14, 9-*cis* violaxanthin and 9-*cis* neoxanthin are abundant in both thylakoid membranes and envelope membranes of plastids and are not water soluble (Rock and Zeevaart 1991, Li and Walton 1990b). The localization of VP14 protein is of great importance in terms of understanding regulation of the cleavage activity and substrate accessibility following stress induction (Tan et al. 1997). Using an plastid protein import system, we show that VP14 is targeted to chloroplasts and predominantly exists in the soluble stroma fraction. A small fraction of VP14 was also found associated with thylakoid.

Proteolysis is an essential process for many aspects of plant physiology and development. These include housing keeping functions such as removing misfolded proteins, generating peptide hormones, processing of organellar and secreted proteins through specific cleavage, and programmed cell death (Vierstra 1996). Protein turnover may also play an important role in both development and environmental regulation of metabolism by reducing the abundance of key enzymes and regulatory proteins. Because VP14 catalyzes a highly regulated step in ABA biosynthetic pathway, and is responsible for the rapid and transit increase of ABA synthesis within hours in water stressed tissues, post transcriptional regulation involving proteolysis is likely a means of regulatory mechanism. Application of cytosolic protein synthesis inhibitor cycloheximide to unstressed bean leaves caused a 50% decrease of ABA within 4 hours after treatment, while treatment of stressed leaves with cycloheximide caused a faster decrease in ABA levels (Li and Walton 1990a). Based on these data, it was predicted that the key steps

controlling ABA synthesis are likely to have a half life of several hours. The enzymatic cleavage of *9-cis* xanthophylls is a good candidate for the regulated step since the downstream enzyme activities are not affected by stress treatment (Zeevaart and Creelman 1988). To address this possibility, the post-translational regulation of VP14 protein was studied in chloroplast. It was found that VP14 is quickly turned over with a half life of less than 30 minutes after import into chloroplast.

Materials and Methods

Plasmid Constructs Higher rates of *in vitro* transcription and translation efficiency have been achieved by shortening the space between the start code and SP6 promoter (Tranel et al. 1995). To remove the 5' untranslated sequence of *Vp14*, the cDNA was amplified from the first ATG with a engineered BamHI site to facilitate the cloning (forward primer 5'-ATGCGGATCCATGCAGGGTCTCGCCCCG, reverse primer T7). The resulting product ligated into pGem-3Z (Promega) under control of the SP6 promoter. The plasmid was named pSP6-*Vp14*.

In Vitro Transcription and Translation pSP6-Vp14 DNA was linearized by digestion in the 3' linker region with EcoRI. The linear DNA was purified from an agarose electrophoresis gel by electroelution. The *in vitro* transcription reaction was carried out as described by Cline et al. (1993).The transcription reaction contained 5 mM DTT, 50 units/ml RNasin (Promega), 0.5 mM NTP's, 50 μg/ml BSA, 1.5mM diguanosine triphosphate [m7G(5')ppp(5')G, Pharmacia]. 50 μg linearized plasmid DNA/ml, 500 units/ml SP6 DNA polymerase in 1 x SP6 DNA polymerase buffer (Promega). The reaction was incubated at 40 °C for 60 minutes. The messenger RNA was precipitated

with ethanol after removing the proteins by phenol/chloroform extraction. The messenger RNA was quantitated and tested for *in vitro* translation efficiency in a wheat germ cell free system (Promega).

A typical 50 µl translation reaction included 30 µl of premade wheat germ cell-free extract, 5 µl premix of all amino acids except leucine, 5 µl 5xBuffer, 5 µl *in vitro* synthesized RNA (~ 40 ng), 5 µl RNase-free water containing ^{3}H-leucine (3000 Ci/mol, DuPont). The mixture was incubated at 25 °C for 1 hour and placed on ice to stop translation. Immediately before the import assay, translation product was diluted with equal volume of 60 mM leucine in 2x import buffer (IB, 50 mM hepes/KOH pH 8 and 0.33 M sorbitol).

Isolation of Pea Chloroplasts Leaves harvested from dwarf peas grown in vermiculite for 10 days at 20 °C, 150 µE fluorescent light were homogenized in a buffer containing 50 mM Hepes/KOH (pH 7.5), 0.33M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM sodium ascorbate and 0.2% BSA. Homogenate was filtered through one layer of miracloth and centrifuged at 2000 g (3200 rpm) for 3 minutes in a Beckman swinging bucket rotor. The pellet was resuspended in grinding buffer (GR) and loaded onto a precentrifuged (50,000 g, 40 minutes) 35% Percoll gradient in GR buffer supplemented with 10 mM glutathione. The gradient was centrifuged at 2000 g for 15 minutes in a swinging bucket rotor and a lower band which contained the intact chloroplasts was removed and diluted 3-fold with IB. The chloroplasts were pelleted at 1500 g and finally resuspended in IB at 1 mg chlorophyll/ml as determined spectrophotometrically.

Protein Import Assay The protein import assay was carried out according to Cline et al. (1993) in the 1 x IB. All the import reactions were carried at 25 °C under light with 5 mM Mg-ATP except where ATP and light dependent import was tested. The unimported proteins were removed by washing the chloroplasts with IB buffer and the surface adhering protein removed by treatment with thermolysin. The chloroplasts were repurified through a 35% percol cushion, centrifugation and rinsed once with 1xIB. The chloroplasts were lysed by suspension in a hypertonic solution containing 20 mM EDTA. For subfractionation of membrane and soluble compartments, chloroplasts were lysed in HKM on ice for 5 to 10 min. Lysis was monitored by using percoll cushion centrifugation to separate the unlysed chloroplasts. Lysed chloroplasts were collected by centrifugation at 4,200 rpm for 8 min. The pellet fraction contained thylakoids and was rinsed twice with 1xIB before lysis for proteins. The supernatant fraction was further centrifuged at 18,000 g for 2 hours at 4 °C to pellet the envelope membrane fraction, the supernatant fraction collected as the stroma fraction. All the subfractions were lysed in 20 mM EDTA and denatured in 15% SDS at 80 °C for 10 minutes, then analyzed in 12% SDS-PAGE. The gel was treated with DMSO and PPO/POPOP then dried. The radioactive protein was detected fluorography (Cline et al. 1993).

Results

The N-Terminal Sequence of VP14 Has Features of a Chloroplast Transit Peptide

Vp14 encodes an 11, 12 *9-cis* epoxy carotenoid dioxygenase that cleaves 9-*cis* violaxanthin and 9-*cis* neoxanthin to produce xanthoxin, a precursor of abscisic acid (ABA) as indicated in Fig. 4-1B (Schwartz et al. 1997, Tan et al. 1997). This cleavage

A

MQGLAPPTSVSIHRHLPARSRARASNSVRFSPRAVSSVPP
AECLQAPFHKPVADLPAPSRKPAAIAVPGHAAAPRKAEGG
KKQLNLFQRAAAAALDAFEEGFVA.....

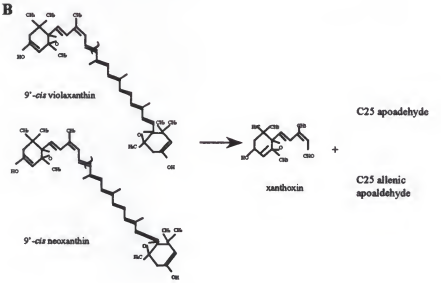


Fig. 4-1. The sequence of 104 amino acid N-terminus extension of VP14 that does not align to LSD and RPE65 (A) and the reactions catalyzed by VP14 in ABA biosynthesis (B). The putative transit peptide region was underlined with single line and a putative amphipathic α-helix region was underlined with a double line.

reaction is the first committed step in the ABA biosynthetic pathway in plants, and substantial evidence has indicated that it is also the regulated step controlling synthesis of ABA. VP14 is homologous to a bacterial lignostilbene dioxygenase (LSD) which catalyzes a very similar cleavage of a conjugated double bond to produce products with -CHO group (Kamoda et al. 1993a, b), and to a human eye retinal epithelium specific protein RPE65 (Hamel et al. 1993). A three way alignment of VP14 with LSD and RPE65 indicates that VP14 has an ~100 amino acid extension at the amino terminus as shown in Fig.4-1A. Blast searches using the extension sequence did not reveal any significant similarity to known proteins. Removal of 30 amino acids from N-terminus does not affect the activity (data not shown), suggesting that this extension may have other functions.

Although no conserved sequence has been found among the chloroplast targeting transit peptides, there are some features shared by the stroma-targeting domains(STD), such as being rich in hydroxylated residues and lacking in acidic residues (Cline and Henry 1996). The sequence of the first 40-50 amino acids at N-terminus were consistent with a STD. It contains 20% percent serine, compared to 5 % for the whole protein, and is positively charged (pH 12.10) in contrast to the full protein which has an isoelectric point of pI 5.72. Similar features are also observed in the N-terminus of ABA2 in tobacco, an epoxidase that is upstream of VP14 in the ABA biosynthetic pathway (Marin et al. 1996). Also noticed was a putative amphipathic α-helix that was highly conserved among a maize homolog and *Arabidopsis* homologs (Deng and McCarty, unpublished data). Amphipathic α-helices are potentially involved in protein-protein interactions or anchoring protein to membranes (Carr et al. 1993).

Chloroplast proteins synthesized in cytoplasm can be tested in an in vitro system in which proteins are radiolabeled during in vitro translation and incubated with intact chloroplasts. Chloroplast targeted proteins will use the protein trafficking pathway to reach their organellar destination which is inaccessible to proteinase treatment. The specific protein can be detected by analyzing the radioactivity of proteins isolated from chloroplasts after proteinase treatment. VP14 was tested for trafficking to chloroplasts using an import assay. To do so, full length *Vp14* cDNA was placed under the control of SP6 promoter in pGEM-3Z (Promega) plasmid, then transcribed and translated *in vitro* using SP6 DNA polymerase and wheat germ cell free systems, respectively. Because the full length *Vp14* cDNA has a 100 nucleotide 5' untranslated sequence in front of the first ATG, a poor translation efficiency was detected. In order to enhance translation efficiency, the 5' UTR was removed by PCR amplification and by subcloning of *Vp14* coding region into the pGEM-3Z plasmid. Upon incubation of *in vitro* translated VP14 with the intact pea chloroplasts, the VP14 protein was imported into a chloroplast compartment that is not accessible to the proteinase thermolysin (Fig 4-2A). In addition, upon import into chloroplasts, VP14 was processed to a smaller molecular weight with the loss of about 4.5 kd. This is consistent with proteolytic cleavage and removal of a N-terminal peptide. The control protein, light-harvesting-complex-protein (LHCP), a well characterized chloroplast thylakoid protein that is involved in photosynthesis, is processed by losing its transit peptide to produce a mature protein of about 28 kd. As indicated in Fig.2B, similar to pLHCP, import of VP14 requires energy provided by either ATP or light which further confirmed the chloroplast localization of VP14. Thus, these data indicate that VP14 is localized in chloroplasts.

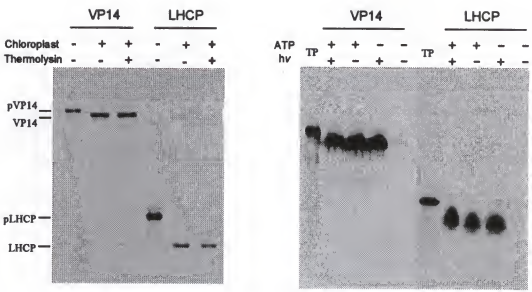


Fig. 4-2. Import of VP14 into chloroplasts and its energy dependence. ^{3}H-leucine labeled precursor VP14 or LHCP were incubated with c hloroplasts and then treated with thermolysin in the presence or absence of ATP or light.

VP14, a Soluble Protein Is Localized in Stroma and Thylakoid Membrane

The next question is where inside the chloroplast VP14 is localized. Chloroplasts have six different compartments including outer and inner envelope membranes, envelope interspace, stroma, thylakoid membrane and thylakoid lumen. Substrates of VP14, *9-cis* neoxanthin and *9-cis* violaxanthin, have been reported in both thylakoid membranes and envelope membranes (Li and Walton 1987, Zeevaart and Creelman 1988, Parry and Hogan 1991). They presumably exist in the membranes through interaction with membrane associated proteins, as found in a carotenoid-protein complex (Markwell et al. 1992). The sub-organelle localization of VP14 reflects to some extent the likely site of interaction between substrates and enzyme. To further locate VP14, chloroplasts were subfractionated into three fractions, the soluble fraction which presumably contains the stromal proteins and interspace, the thylakoid fraction that includes all thylakoid membrane and lumen proteins, and envelope fraction which includes outer and inner envelope proteins. VP14 was found predominantly in the soluble fraction, suggesting that it may be a stroma protein (Fig. 4-3). Moreover, there was a significant amount of VP14 associated with thylakoid. Further treatment of the thylakoid with thermolysin digested VP14, indicating that the protein was bound on the outer face of thylakoid membrane. LHCP, a thylakoid membrane protein was partially protected from thermolysin digestion consistent with the evidence that the protein is embedded in the membrane so that it is not accessible to an external proteinase (Fig. 4-3).

Evidence of Fast Turnover of Imported VP14

In the import assays, it was noticed that small sized radiolabeled proteins were also

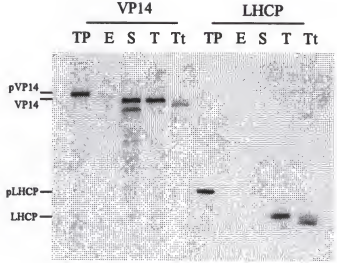


Fig. 4-3. Localization of mature VP14 inside the chloroplast. After incubated with ^{3}H-pVP14, purified chloroplasts were lysed and separated into subfractions of stroma (S), thylakoids (T) and envelopes (E). An equal amount of thylakoids were treated with thermolysin (Tt).

detected in the soluble fraction, which we hypothesized might be degradation fragments of VP14. Because stress induction of ABA synthesis is transient, the presumed key regulated step, the dioxygenase may be subject to rapid regulation at both the transcription level and protein level. We have detected using both monoclonal and polyclonal antibodies that VP14 protein exists at very low levels in even stressed leaves of maize in which *Vp14* transcripts were found increased significantly and ABA levels were increased about 40-fold (Data not shown). One possibility is that VP14 may undergo fast turnover such that the steady state level is low. Using the import assay, we measured the rate of turnover of mature VP14 in isolated chloroplasts compared to a control thylakoid protein, LHCP. After the import, precursors were removed by thermolysin which was subsequently removed by repurifying the chloroplasts. The chloroplasts were incubated at 28 °C under light for different periods (Materials and Methods), the level of the imported mature VP14 was determined. As shown in Fig. 4-4A, level of imported VP14 was quickly declined within 60 minutes, while no significant decrease in LHCP was observed over the same period time (data not shown).

It has been reported that mis-targeted and misfolded proteins are likely to be degraded in chloroplasts (Halperin and Adam 1996, Vierstra 1996). As VP14 was found both in the soluble and thylakoid fractions, we examined the degradation pattern in both fractions. The degradation of VP14 was about the same in either the soluble fraction and the thylakoid fraction (Fig. 4-4B). Because VP14 requires a non-heme iron in its active form (chapter 3), we considered the possibility that iron may be needed to stabilize the protein. To test this, ferrous iron and ferric iron were supplied to the chloroplasts before

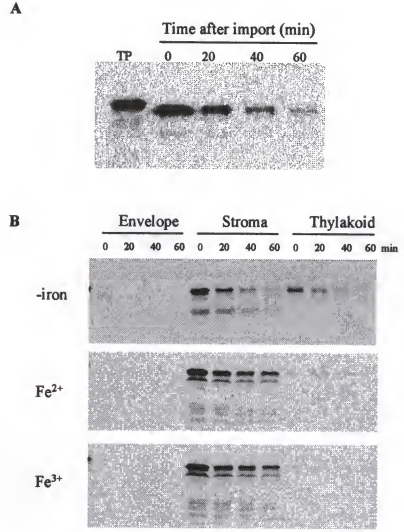


Fig. 4-4. VP14 is rapidly degraded after import into the pea chloroplasts. VP14 was translated *in vitro* in presence of ^{3}H-leucine and imported into chloroplasts. Then the precursor was washed off and the chloroplasts were tested for time-course of transport. A. whole chloroplasts; B. fractionated chloroplasts; TP=translation precursor.

import of VP14. VP14 was still subjected to degradation after addition of iron (Fe^{+2}, Fe^{+3}), although it seemed that Fe^{+2} may stabilize VP14 to some extent.

Discussion

VP14 Is Localized in Both Stroma and Thylakoid Membrane

The N-terminal sequence of VP14 showed certain features of a stromal targeting domain (STD) such as a high percentage of hydroxylated amino acids and positively charged amino acids as reviewed by Cline and Henry (1996). *In vitro* synthesized VP14 was imported into isolated pea chloroplasts and localized in the soluble stromal fraction and thylakoid membrane fraction (Fig. 4-3). The soluble fraction was considered to contain proteins mainly from the stroma, but proteins that exist between two envelope membranes could potentially be released in this fraction during chloroplast lysis. In fact, there was a faint band detected in envelope fraction (Fig. 4-3), which was likely due to the contamination by thylakoids, because a faint band was also observed in envelope fraction in the control of stroma localized small subunit of LHPC. Experimentally it is difficult to isolate envelopes absolutely free of thylakoid contamination. A small amount of thylakoids lysed during experiment produces membrane vesicles that are inseparable from envelopes. However, the abundant presence of VP14 in the soluble fraction but not with envelopes favors a stromal localization of VP14.

The sequence of VP14 has a putative amphipathic α-helix which is also conserved in its homologs in *Arabidopsis* (Deng, McCarty, Unpublished data). Reports have indicated that such kinds of protein structure have been implicated in protein-protein interaction or anchoring proteins onto membranes (Carr 1991). The substrates of VP14,

9-*cis* neoxanthin and 9-*cis* violaxanthin are found chiefly in envelopes and thylakoids membranes. Thus anchoring of the soluble VP14 protein could be essentially to make it accessible to either one or both substrates. Biochemical labeling studies using ^{14}C-MVA indicate that separate pools of carotenoids are used for ABA biosynthesis (Parry and Horgan 1991). VP14 was found in association with thylakoid membrane with a topology of facing stroma, as indicated by its accessibility by thermolysin (Fig 4-3B). Based on this indirect evidence, we propose that the localization of VP14 in thylakoid membranes is possibly regulated by environmental and developmental factors. The amphipathic α-helical domain could anchor the protein to the membrane and provide to access the membrane localized substrates. With immunolocalization of VP14 by monoclonal antibody, this idea will be investigated in later studies.

Post-translational Regulation Is One of the Mechanisms for its Regulation

The import assay of VP14 revealed rapid turnover of mature VP14 inside the chloroplasts. We identified the fast turnover as a possible regulatory mechanism in plants. As discussed previously, the dioxygenase encoded by *Vp14* is considered to the regulated step in ABA biosynthetic pathway based several lines of evidence (refer to chapter 3). The relative abundance of its substrates and the constitutively active state of the downstream enzymes that convert xanthoxin to ABA make the oxidative cleavage reaction the regulatory step in the pathway. The ways in which environmental and developmental factors regulate VP14 was not known. Our preliminary data indicated that the VP14 level was extremely low in even stressed maize leaves, even though Vp14 steady state transcript levels were increased dramatically by water stress. Because ABA levels increase by up to

40-fold in stressed leaves (Wright and Hiron 1969, Chandler and Robertson 1994), and re-watering can quickly restore the synthesis to normal (Ingram and Bartels 1996), the de novo synthesized VP14 is likely to be degraded or inactivated quickly such that it does not lead to ABA production. Studies which show inhibition of ABA biosynthesis by cycloheximide indicate that a key regulated enzyme in ABA biosynthetic pathway could have a half life as short as 1 hour (Li and Walton 1990a). Consistent with that, we found that VP14 was quickly degraded inside the chloroplasts with a half life about 30 minute in the isolated chloroplasts, and this fast turn-over rate was not likely a result of either mis-targeted or abnormally folded protein. Addition of ferrous iron can result in a correct folding of VP14, thus activate the cleavage activity (Chapter 3). However, Fe3+ did not prevent degradation of VP14. The fast turnover is also unlikely due to a heterologous environment for VP14, a maize protein in pea chloroplast, as a number of such cases have been reported that do not turnover rapidly (Cline and Henry 1996). Thus, we suggest that fast turn over of VP14 is a possible regulatory mechanism controlling ABA biosynthesis is plants.

CHAPTER 5
A FUNCTIONAL DUPLICATE OF *VIVIPAROUS14* CONFERS A DISTINCTIVE TISSUE-SPECIFIC EXPRESSION PATTERN IN MAIZE

Introduction

Abscisic acid (ABA) is synthesized by oxidative cleavage of 9-*cis* xanthophylls to form xanthoxin, which is subsequently converted to ABA. The *vp14* mutant selectively blocks ABA synthesis in the developing seed and suggests that the *Vp14* gene has a role in developmental control of hormone synthesis in maize. *Vp14* encodes the dioxygenase enzyme responsible for oxidative cleavage of 9-*cis* xanthophylls to xanthoxin (Schwartz et al. 1997) , which is believed to be the key regulated step in the ABA biosynthetic pathway (Zeevaart and Creelman 1988, Walton and Li 1995). Biochemical analysis of the mutants indicated that there is about a 70% reduction of ABA in developing embryos, whereas no reduction of ABA levels was found in other tissues including leaves and roots (Tan, et al. 1997). Two possible explanations may account for the residual ABA in *vp14* mutants: 1) The existing *vp14* mutant alleles are leaky, 2) Functionally redundant *Vp14*-like genes exist. A leaky expression of a functional VP14 protein from the *vp14-2274* allele would likely require splicing of the *Mu1* insertion from the transcript. We detect little evidence of wild type size transcripts in mutant embryos on northern blots (Tan et al. 1997, refer to chapter 2). Furthermore, it is hard to explain that the *vp14-2274* mutation can specifically affect ABA biosynthesis in embryos and retain normal function in other tissues if *vp14-2274* is leaky. Several lines of indirect evidence favor the second possibility. First, the

observation that *vp14* does not markedly affect ABA levels in vegetative tissues and that mutant plants develop normally under field conditions implies strongly that the *Vp14* gene accounts for only a subset of the ABA biosynthetic activity in the plant. Thus, partial suppression of the *vp14* phenotype by ABA transferred from maternal tissues might explain the variable penetrance of the viviparous phenotype we observe under field conditions. Ober and Setter (1992) reported that maternal ABA synthesis induced by stress can significantly affect seed development in maize. Secondly, detached *vp14* leaves accumulate ABA nearly as much as the wild type, suggesting the expression of a functionally active dioxygenase in stressed leaves. Thirdly, we detected evidence of related genes on moderate stringency Southern and northern blots. In addition, several related sequences are found in the rice, maize and Arabidopsis expressed sequence tag (EST) collections. Thus, the evidence points to overlapping expression of functionally equivalent *Vp14*-like genes with different patterns of tissue specific expression.

Here we reported the cloning and sequencing of a highly homologous *Vp14* duplicate gene (*Ecd1*) that was mapped to short arm of chromosome 5. The distinctive expression pattern of *Ecd1* relative to *Vp14* provided evidence for a potentially important mechanism in regulating hormone biosynthesis in higher plants.

Materials and Methods

The plant genotypes used and growth conditions were described as in Chapter 2.

Southern Blot and Northern Blot Analysis As described in Chapter 2.

Construction and Screening of Clone Libraries For cloning of the 3.2 kb EcoRI fragment identified by hybridization to the *Vp14* conserved probe (P2), about 100 μg

genomic DNA was digested with EcoRI to completion, then size fractionated on a 10-40% sucrose in TE (pH 8.0) gradient. The gradient was centrifuged at 85,000 g in a Beckman SW41 rotor at 4 °C for 24 hours. DNA in each fraction was precipitated in 70% ethanol and the fractions tested by Southern blot to locate the fraction containing the 3.2 kb fragment. DNA in this fraction was ligated to λ-ZAP (Stratagene) arms and packaged into lambda phage. The library was screened with the *Vp14* P2 probe (refer to Fig. 2-4 in Chapter 2). The resulted lambda phage was *in vivo* excised by a helper phage to yield a Bluescript plasmid containing 3.2 kb EcoRI fragment, named *pEcd1*.

Water Stress Treatment Leaves of well watered maize leaves of about 2 week of age were detached and put on the lab bench at room temperature until they lost 15% of their fresh weight to transpiration. Then they were sealed in plastic bags and kept at room temperature for 6 hours. Leaves then were frozen in liquid nitrogen and stored at -80 °C until use.

Results

Molecular and biochemical analysis of *vp14* mutants indicate that *vp14* is specifically blocked in the epoxy-carotenoid cleavage in the ABA biosynthesis in seeds (Tan et al. 1997). The expressed recombinant VP14 protein was shown to specifically cleave the 11,12 double bond of 9'-*cis* neoxanthin and 9-*cis* violaxanthin to generate xanthoxin as indicated in Fig. 5-1A (Schwartz et al. 1997). Southern blot analysis using the conserved sequence of *Vp14* (P2, refer to Chapter 2) revealed multiple EcoRI fragments in the inbred W22 and Q69 genomic DNAs, suggesting that *Vp14* belongs to a gene family (Fig. 5-1B). In support for this, several *Vp14*-like sequences have been

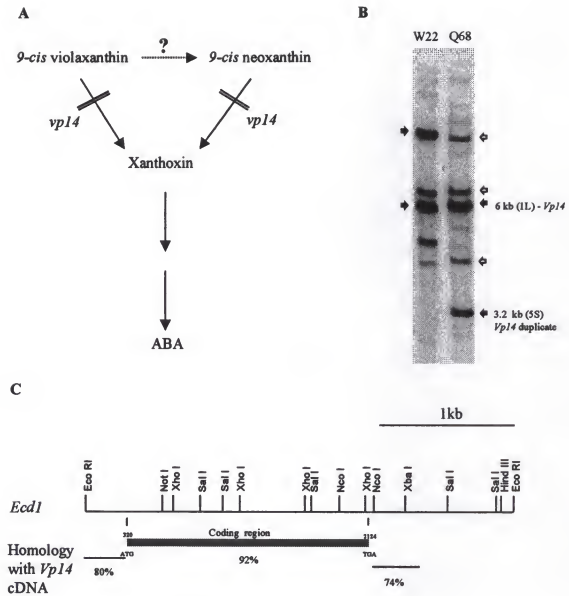


Fig. 5-1. Cloning of a *Vp14* duplicate, *Ecd1* in maize. **A**. The blocked step of ABA biosynthesis in *vp14* mutant. **B**. A low stringency Southern analysis of W22 and Q68 DNA with a probe (P2) of a highly conserved region of *Vp14*. **C**. Restriction map of the cloned 3.2 kb EcoRI fragment and the open reading frame. The homologous region with *Vp14* cDNA was marked.

recovered from genomic and cDNA libraries (Data not shown). A 3.2 kb EcoRI fragment in Q69, which was polymorphic in inbred W22, showed an almost equal hybridization intensity as *Vp14*, suggested that it may contain a closely *Vp14*-related sequence. To search genes that potentially encode proteins functionally equivalent to VP14, this fragment was cloned from a subgenomic library constructed from enriched 3.2 kb EcoRI fragments (See Materials and Methods). Sequencing of this fragment revealed a strong homology to *Vp14* cDNA (Fig. 5-1C). This clone, designated *Ecd1* for potentially encoding an epoxy-carotenoid dioxygenase like *Vp14*, suggested an identical gene structure as compared to *Vp14*. The DNA alignment with *Vp14* cDNA indicated that the coding region is not interrupted by any introns, a feature of *Vp14* but quite rare in other eukaryotic genes. The position of the open reading frame and a putative TATA box were highly conserved between *Vp14* and *Ecd1* (Fig. 5-2). The coding sequence had a 92% identity to *Vp14* at nucleotide level, indicating that *Ecd1* is a recent duplication of *Vp14*. The lower homology to *Vp14* at the 5' and 3' flanking sequences hinted at a diversification of expression during evolution.

This clone was mapped by the Missouri mapping center to a location between RFLP markers *umc83b* and *csu222b* on the short arm of chromosome 5. There is accumulating RFLP evidence that a segment of the maize chromosome 1L which include the *Vp14* gene, was duplicated during the evolution of maize (Helentjaris et al. 1988). Molecular clock analysis based on synomynous nucleotide substitutions in the *Vp14* and *Ecd1* coding regions indicates that the duplication occurred 12.6±0.5 million years ago (J. Doebley, unpublished). This is in line with evidence of other duplications in maize such as

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C1 and *Pl* (Cone et al. 1993). The open reading frame of *Ecd1* encodes a 601 amino acid protein with a calculated molecular mass of 65.5 kd, very close to VP14 (Fig. 5-2).

The alignment of the deduced protein sequence of ECD1 with VP14 provided further evidence that ECD1 may function as an VP14-like epoxy-carotenoid dioxygenase. The two proteins share an identity of 93% of amino acid sequences, and a similarity of 99% (Fig. 5-3). Gaps in *Ecd1* only include a triple amino acid (KPV) deletion close to the possible cleavage site of the transit peptide (refer to Chapter 4), and one missing alanine residue at the C-terminus of the protein. In a multiple sequence alignment with closely related proteins found in the database, the conserved histidine and tyrosine residues including the surrounding clusters of sequences present in the VP14, LSD, REP65, SYN1 and SYN2 proteins (Chapter 2) are also conserved in ECD1. Those residues are suggested to chelate the co-factor iron which is proven to be critical to the activity of VP14 (Chapter 3). Features of a putative transit peptide, a high percentage of hydroxylated amino acids (serine is 20% in the first 50 amino acids) and positively charged residues are also conserved. In addition, a putative amphipathic α-helix appears to be highly conserved between ECD1 and VP14 (boxed region in Fig. 5-3). These features strongly support the idea that *Ecd1* is a *Vp14* duplicate.

The high degree of identity between ECD1 and VP14 proteins strongly indicated the ECD1 may have the same function as VP14, *i.e.* cleavage of 9-*cis* neoxanthin and 9-*cis* violaxanthin, provided that *Ecd1* is expressed in plants. Furthermore, if at least two functionally identical enzymes (isoenzymes) exist in maize, it would be of great interest to know how they are regulated or coordinated throughout plant developmental processes. The expression pattern of *Ecd1* was studied by northern blot analysis and compared to

Fig. 5-3. Alignment of the VP14 duplicate with VP14. * indicates identical; - gap, ^ conserved histidine residues in LSD, REP65, SYN1 and SYN2. Underlined region refers to a putative transit peptide. Boxed region refers to a putative amphipathic α-helix.

Vp14 in both water stressed and nonstressed leaves. Because *Vp14* and *Ecd1* were highly homologous in the coding region, a less conserved 3' untranslated sequence was used as gene specific probe for *Ecd1*. To avoid ambiguity in the event that *Vp14* and *Ecd1* may have roughly equal sized mRNA's and cross hybridization, *vp14* homozygous mutants were used as a positive control for the *Vp14* transcript since a *Mu1* insertion in the coding region resulted in a 4.0 kb transcript (Fig. 2-8, Chapter 2). As indicated in northern blot analysis in Fig. 5-4, there were four bands detected in the water stressed leaves of *vp14* mutant by a *Vp14* coding sequence probe, P2 (the probe was illustrated in Fig. 2-4C, chapter II). The 4.0kb marks the *vp14* message containing a 1.4 kb *Mu1* insertion. *Vp14* messages were induced in water stressed leaves, whereas little was detected in the unstressed leaves. In addition, two bands smaller than the *Vp14* transcript (2.6 kb) were evidently detected by this probe, indicating the expression of related genes. It is important to keep in mind that *Vp14* and *Ecd1* have a 95% identity in nucleotide sequence in the P2 region. When the same blot was hybridized with the 3' untranslated sequence of the *Ecd1* genomic DNA which has a 74% identity to *Vp14* as indicated in Fig. 5-1C, a little cross hybridization to the 4.0 kb band in *vp14-2274* and *vp14-3250* was expected. However, a strong hybridization to one of the smaller bands (~ 1.5 kb) was detected. The intensity strongly suggested that it is a transcript from the *Ecd1* gene which may be spliced or transcribed from an alternative start site. The sequence of this smaller size transcript is currently under investigation. The weak hybridization to P2 probe suggested that this transcript may not have an intact P2 corresponding region as indicated in the genomic ORF. Furthermore, this smaller transcript appeared to be expressed constitutively in leaves

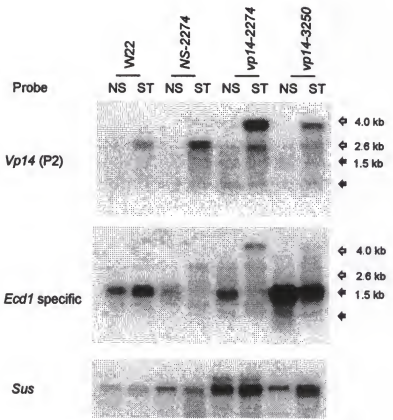

Fig. 5-4. Expression pattern of *Vp14* and *Ecd1*in water stressed (ST) and non-stressed (NS) leaves of inbred W22, *Vp14* and two *vp14* alleles. The same blot was hybridized with three different probes. Each lane was loaded with roughly 500 ng poly(A)-RNA.

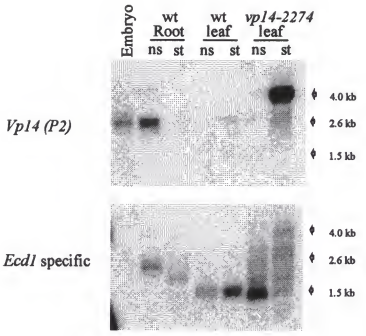

Fig. 5-5. Northern blot analysis of *Ecd1* expression in embryos and stressed roots. st, water stressed; ns, nonstressed.

and not affected by water stress. The reason for the lack of this smaller message in stressed homozygous *vp14-2274* mutants was not known.

Similarly, the expression of *Ecd1* was explored in developing embryos and roots. Because *Vp14* was found to be expressed in both tissues (Tan et al. 1997, Chapter 2), the stressed mutant *vp14-2274* was used as a control for *Vp14* cross hybridization when *Ecd1* specific probe was used. As shown in Fig. 5-5, under a high stringency, the P2 probe hybridized to *Vp14* transcripts shown in embryos and nonstressed roots as 2.6 kb of wild type size, and in water stressed *vp14-2274* leaves as 4.0 kb of mutated form as shown previously (Tan et al. 1997). Surprisingly, P2 did not detect any signal in the water stressed roots, suggesting that at least *Vp14* expression is down-regulated in stressed roots. This is markedly different from *Vp14* expression in leaves where it is strongly water stress inducible as also indicated in this blot and previously (Tan et al. 1997). As mentioned above, the P2 probe may also recognize *Ecd1* based on 95% identity in the *Ecd1* genomic sequence provided that the *Ecd1* transcript is the same size as *Vp14*. When the same blot was probed by the *Ecd1* specific probe, three bands of different sizes were detected (Fig. 5-5). A 2.6 kb transcript was detected present in the nonstressed roots, and a transcript between the 2.6 and 1.5 kb in stressed roots. The barely visible of the 2.6 kb signal in the embryo and faint signal of the 4.0 kb transcript which seems to be overloaded, argue that the 2.6 kb transcript present in nonstressed roots is *Ecd1*.

Discussion

The biochemical analysis of mutant *vp14* and molecular cloning of *Vp14* gene led to a suggestion that multiple functionally identical but differentially expressed *Vp14* related

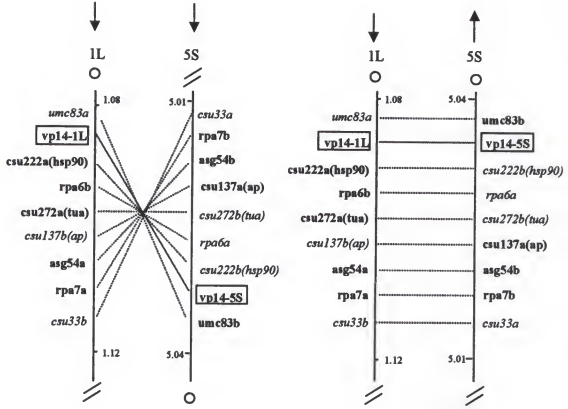

Fig. 5-6. Maps of chromosomes 1L and 5S showing locations of duplicated markers. Only markers that are duplicated on both chromosomes are indicated. The order of the markers is according to MMN (1996), while the distance between markers was not precisely indicated. Centromeres were marked with circles. Arrows indicate orientations of the chromosomes.

genes may control the biosynthesis of ABA throughout plant development. Different patterns of tissue specific expression have been found in a number of gene families.

Differentially regulated gene families are emerging as an important mechanism underlying developmental control of biochemical pathways in plants. The ACC synthase genes that encode the key regulated step in ethylene hormone biosynthesis also form differentially regulated gene family in plants (Liang et al. 1989). It remains to be seen whether key steps in other hormone synthetic pathways will have this feature. The duplication feature of *Vp14* in chromosome 5S may be specific to maize. There is a large body of RFLP evidence of a duplicated chromosome segment on 1L and 5S of maize that is presumably a residue of a tetraploid ancestry (Helentjaris et al. 1988). In an analysis of existing RFLP markers neighboring *Vp14* and its duplicate *Ecd1* on chromosome 1L and 5S respectively (Maize Genetics Cooperation Newsletter 70 1996), we found clusters of conserved RFLP sequences between the two (Fig. 5-6). The order of the markers suggested that duplication is inverted between 1L and 5S.

The highly conserved gene structure and sequence between *Vp14* at 1L and *Ecd1* at 5S provided additional molecular evidence for such a duplication. The 93% identity in amino acid sequence indicates that the two proteins are likely to have the same enzymatic activity, *i.e.* cleaving 9'-*cis* neoxanthin and 9-*cis* violaxanthin to produce xanthoxin in the ABA biosynthetic pathway. The distinctly different pattern of *Ecd1* expression compared to *Vp14* provides an explanation for the normal levels of ABA in tissues like roots, and perhaps the residual ABA detected in embryos because low level expression can not be ruled out by northern bolt analysis. The relatively divergent presumptive promoter region of *Ecd1* may substantiate the tissue specific expression. By analogy to other duplicate

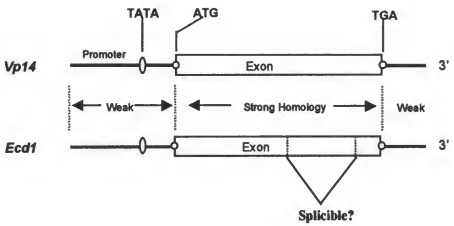

Fig. 5-7. Diagram of *Ecd1* and *Vp14* gene structure and possible splcing in *Ecd1*.

gene pairs that have been analyzed in maize (Chandler et. al. 1989, Cone et. al. 1993), we believe that *Vp14* and *Ecd1* have evolved distinctive patterns of tissue specific gene expression. Instead of having a complex elements in the promoter of a key gene in a pathway, plants may choose simple divergent promoters for a family of functionally related genes to achieve the regulation and integration of developmental and environmental signals.

As to how important *Ecd1* is to the plant remains to be determined. The absence or reduced level of *Ecd1* transcripts in *vp14-2274* is unexplained. One possibility is that the tested line of *vp14-2274* is a double mutant of *vp14* and *ecd1* such that it tends to be more penetrant than *vp14-3250*. An outcross of this specific line to inbred like W22 should resolve this question. If it is a double mutant, and then the *Ecd1* may also only contribute another small subset of ABA in plants like *Vp14* does. It may be possible that other *Vp14* related genes besides *Ecd1* might encode proteins functionally equivalent to VP14. *Vp14* homologous cDNA's recovered from roots and another clone from embryos may be the candidates for such genes.

An interesting finding is that in leaves the transcript detected by a probe of 3' untranslated region of *Ecd1* is smaller than *Vp14*. That probe has a 60% homology to *Vp14* which is evidently reflected by the weak hybridization to *Vp14* mRNAs (4.0 and 2.6 kb, Fig. 5-4), suggesting that the strong signal is very likely the *Ecd1* transcript. One possibility is that an internal sequence was spliced out as an intron as shown in the diagram (Fig. 5-7). As hybridization of the conserved 1 kb P2 probe to the smaller sized *Ecd1* transcript was much weaker than to *Vp14*, and the homology of that region is as high as 95% at nucleotide level, it appears that the smaller transcript might have a spliced

sequence in that region, such as an intron. Based on the sequence alignment of VP14 with *Ecd1*, such a spliced massage would not be translated into a functional enzyme. It is possible that the splicing of an internal sequence in the coding region of *Ecd1* might play a role in the regulation of the expression of that gene, and that splicing may be developmentally and environmentally regulated. However, the northern blot analysis with 3' sequence probe of *Ecd1* did not detect any significant increase in the 2.6 kb message, suggesting that there is not a significant increase in the unspliced version at least in dehydrated leaves. Further cloning of the cDNA and northern analysis using the splicing sequence as a probe should provide more insights to that question.

SUMMARY AND CONCLUSIONS

By searching for viable and green viviparous mutants from Robertson's mutator lines, we have isolated a new ABA deficient mutant, *viviparous14* (*vp14*) that specifically blocks ABA biosynthesis in seeds. It was proven to be an important mutant as biochemical analysis indicated that *vp14* is blocked in the oxidative cleavage of 9-*cis* xanthophylls, the first committed step in the ABA biosynthetic pathway. *Vp14* was cloned by transposon tagging and the sequence shares strong homologies to lignostilbene dioxygenase of *pseudomonas* and RPE65 of mammals. VP14 as expressed in *E. coli* can specifically cleave 9'-*cis* neoxanthin and 9-*cis* violaxanthin to produce xanthoxin, an immediate precursor of ABA. VP14 was synthesized as a precursor and processed upon import into chloroplasts. *Vp14* belongs to a gene family that potentially plays a role in the coordinated regulation of ABA biosynthesis in different tissues at different developmental stages and in response to environmental signals. A duplicate of *Vp14* was cloned and it may encode a protein functionally equivalent to VP14, which is expressed with a distinctly different in development. VP14 is subject to fast turnover (half life ~30 minutes) which taken together suggested that expression of *Vp14* may be regulated transcriptionally and post-transcriptionally.

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I certify that I have read this study and that in my opinion it conform to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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